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Award Number: DAMD17-00-1-0053

TITLE: Development of Genetic Therapies for the Hemidesmosol Subtypes of Junction Epidermolysis Bullosa

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REPORT DATE: November 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 2003	Final (1 Nov 1999 - 31 Oct 2003)

4. TITLE AND SUBTITLE
Development of Genetic Therapies for the Hemidesmosol Subtypes of Junction Epidermolysis Bullosa

5. FUNDING NUMBERS
DAMD17-00-1-0053

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

During the final award period of this project, we focused on creating the model systems for the in vitro gene therapy experiments, in particular, the in vitro skin model. We worked extensively on development of the in vitro skin model, and succeeded in generating recombinant skin between keratinocytes and dermal cells in skin equivalents. To extend these studies and make them ore widely applicable for wound care enhancement in blistering diseases as well as in chemically induced wounding, we then went on to develop a model for in vitro epithelial reprogramming, in which we have begun to utilize different epithelial cell types as donor cells, in addition to keratinocytes, specifically, amnion and cornea cells. Collectively, we have shown that the use of gene delivery combined with epithelial cell-skin equivalent models, show significant promise toward developing a cellular 'bandage' for both genetically and chemically induced skin blistering.

14. SUBJECT TERMS
Epidermolysis bullosa, blister, wound healing, gene therapy, keratinocyte

15. NUMBER OF PAGES
108

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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4. Introduction

In recent years, several lines of evidence about the plasticity of stem cells (Lagasse et al, 2000; Lake et al, 2000; Morrison, 2001; Blau et al, 2001) has prompted our laboratory to consider a novel approach **to the treatment of inherited skin disorders such as EB, as well as in the treatment of chemically induced burns from sulfur mustard.** As in other fields, gene therapy in the skin has been hampered by the inability to target (or identify) a stem cell, and the lack of sustained gene expression. Rather than focusing on the identification and targeting of a stem cell in the skin using exogenous genes, in the final period of this award, **we initiated a new line of experimentation aimed at testing the hypothesis that epithelial cells from other sources might be coaxed into becoming skin cells given the right microenvironment.** Recently, for example, it was shown that adult rabbit cornea cells can be reprogrammed into skin under the right inductive dermal influences (Ferraris et al, 2000). Based on this evidence and other experiments performed throughout this award, **we have begun to define the conditions under which reprogrammed cells might serve as a source of donor cells for therapy in genetic skin disorders and in the treatment of chemical burns.**

During this award, we have developed an *in vitro* model using dermis as the inductive source, and have since demonstrated that we can induce skin-specific keratin gene expression from several primitive epithelia, such as amnion and cornea. In the final award period, **we focused upon the refinement of this model and determination of the pattern and chronology of gene expression changes corresponding to the reprogramming of amnion or cornea into epidermis.** We have successfully reprogrammed several donor epithelia into skin, and have thereby begun to capture the utility of reprogrammed skin for clinical usage in the setting of wound healing and for genetic diseases such as epidermolysis bullosa.

We have also begun to test whether we can recapitulate the *in vitro* results in an animal model. We will test this approach using an *in vivo* model first with immunodeficient mice, and finally with wild-type recipients to determine whether the grafts can be induced *in vivo.* Importantly, we will learn whether any rejection occurs between matched and unmatched donors and recipients, respectively.

The field of gene replacement therapy has evolved rapidly during the past 5 years, and attention in the field has turned toward using grafted donor cells rather than using the patients' own cells that have been genetically corrected. **This approach would offer significant advantages over the conventional gene replacement strategies that were proposed originally, which are currently dependent on the introduction of the exogenous gene as well as the ability to generate long-term expression and engraftment via stem cells.** If these studies are successful, in the future we plan to ask whether the *in vivo* results can be replicated in normal human subjects. Historically, both amnion and cornea have been used safely in site-specific transplantation studies between unmatched individuals without rejection. Using donor cells from skin or other body sites of immunologically compatible healthy individuals would overcome both of these obstacles, since the **gene of interest would already be present,** and the exogenous tissue would be induced to form new skin tissue, and in so doing, **sequester a new population of stem cells.** We have successfully generated both epidermis and hair follicles using cornea and amniotic epithelium as the donor source, and in so doing, we expect that a new niche of stem cells in the hair follicle 'bulge' region has been formed, and will continually repopulate the new skin. This work will continue as we go further in defining the cellular properties of reprogrammed skin.

We believe the concept of cellular transplantation and reprogramming holds great promise toward the eventual goal of successfully treating a broad spectrum of genetic disorders of the skin, including EB, as well as the development of universal bandages for use in chemically-induced or other types of burns. In the final award period, we have proven the feasibility of the concept that donor epithelia can be induced to form skin, laying the foundation for clinical trials of this approach in human wounds and genetic skin diseases such as epidermolysis bullosa.

Background Studies

The major emphasis of many laboratories in epidermal biology, including our own, is on developing gene therapy approaches for skin diseases. As in other fields, gene therapy in the skin has been hampered by: 1) the inability to target (or identify) a stem cell, and 2) the lack of sustained gene expression. Despite numerous efforts by many laboratories in different fields, little progress has been made in overcoming these two obstacles using conventional approaches, and the field as a whole has been moving toward the approach of cell-based therapies and tissue engineering. Along these lines, we therefore asked whether we could identify an ectopic source of epithelial cells that could be induced into becoming a skin stem cell. **Rather than searching for markers of the epidermal stem cell itself, we asked whether we could reprogram other epithelia into skin under the appropriate inductive (dermal) influences.** Two such candidate epithelial tissues are the cornea and amnion, since both have been used extensively in transplantation studies in the past, and since existing evidence suggests that both tissues demonstrate plasticity and the ability to be reprogrammed.

Much work is currently focused on using the skin as a donor tissue of stem cells for other diseases (neurological, muscular) (Toma et al, 2001), however, little interest is focused

on **how to induce other cell types to become skin.** We reasoned that if the donor cells were taken from an immunologically-compatible individual or did not elicit an immune response, **such cells could overcome the two major obstacles in gene therapy approaches: gene introduction and targeting the stem cell.** Donor cells, by definition, would contain an intact gene-of-interest, and importantly, others have shown that epidermal stem cells would be sequestered during the induction of the new skin and hair follicle, thus providing a lifelong supply of genetically corrected cells.

Use of Amnion in Transplantation Biology

Amnion has been widely reported as a biological dressing for burns and its advantages have been well-documented in the literature (Maral et al, 1999, Ruzczak and Schwartz, 2000). It eliminates pain, allows wounds to dry faster and promotes early epithelialization. **Amnion is inexpensive to use, easily obtained and stored, it has antimicrobial properties and low antigenicity, all of which contribute to its utility as a wound dressing,** particularly in developing countries. Several different preparations of amnion have been used, including fresh, frozen, dried, irradiated, lyophilized and glycerolized. In one recent study, **preserved amnion was applied to split-thickness skin graft donor sites in five patients** (Maral et al, 1999). Wounds were covered with nonadherent gauze and left undisturbed, and the wounds were completely epithelialized after 10 days. **Importantly, no evidence of acute or late rejection was observed in any of the subjects (Maral et al, 1999).**

In addition to its application as a wound covering, amnion as well human amniotic epithelial cells (HAE) themselves have attracted interest as potential donor cells for the treatment of metabolic disease (Akle et al, 1981, 1985; Scaggiante et al, 1987). In particular, they were tested as donor cells for treatment of lysosomal storage disorders

including Niemann-Pick Disease and mucopolysaccharoidoses such as Hunter's and Hurler's Diseases. In both studies, repeated implantation of amnion or HAE cells was used as a source of enzyme replacement, and no evidence of immune response toward the transplanted cells was observed.

Historically, the question of the immunogenicity of transplanted amnion has been the subject of much study. It has been shown that HLA-A, B, C and DR antigens are not expressed on freshly collected or cultured HAE cells (Adinolfi et al, 1982; Yeh et al, 1983). Transplantation of amnion into allogeneic hosts does not result in overt acute graft rejection. In one study, HAE were implanted subcutaneously into the arm of seven volunteers, and again, no evidence of acute reaction was present, along with no pain or redness of the skin (Akle et al, 1981). If there was any immune response to the implants, it was low grade and chronic rather than active. In addition, it did not result in rejection of the HAE cells, since they appeared to survive, and in some cases, even proliferate beneath the skin.

Amnion has also been used as a biomaterial in a number of different surgical applications. In microvascular surgery, human amnion has been studied as an acceptable substitute to autologous vein (Gray et al, 1987). Amnion-derived interpositional grafts were shown to have a patency rate similar to that of autologous vein grafts, healed and re-endothelialized within 3-4 weeks, and importantly, were not rejected by the recipient rats. Amnion has also been used in the surgical treatment of congenital absence of the vagina in a series of 21 patients (Tozum, 1976). The transplanted amnion was found to form a mitotically active, proliferating squamous vaginal epithelium. The authors report that "(Amnion) has a superb capability for regeneration and metaplasia. Since it is embryonic tissue which activates proliferation and regeneration of the cells adjacent to it, it is accepted and

not rejected by the recipient. Eventually these cells respond to exogenous estrogens by mitosis and maturation of an epithelium" (Tozum, 1976). Finally, HAE cells have been used as donor cells for reestablishment of a damaged ocular surface (He et al, 1999). Following transplantation of HAE cells onto denuded corneas, they were found to repolarize and tightly adhere to the underlying stroma via newly formed hemidesmosomes, suggesting they are capable of generating specialized keratinocyte-like attachement junctions.

More recently, HAE cells have received renewed attention in cellular therapy because of emerging evidence about the general plasticity of stem cells (Morrison, 2001; Blau et al, 2001). The amnion is the inner layer of the fetal membranes and is contiguous with the ectoderm of the embryo. It is composed of a single layer of cuboidal or flattened epithelial cells on the inner surface and a mesenchymal connective tissue layer on the outside. At about 8 days after fertilization in humans, a small cavity appears within the epiblast that enlarges to become the amniotic cavity. HAE cells are formed from amnioblasts adjacent to the cytotrophoblasts, and line the amniotic cavity as well as the rest of the epiblast. For this reason, **the hypothesis has been put forward that HAE cells may have the potentiality to differentiate into various organs including the heart, brain and liver, given the correct microenvironment** (Sakuragawa et al, 1996). Several studies have since shown that HAE cells may have the putative multipotentiality of neurons, astrocytes and oligodendrocytes, and express markers for both neuronal and glial cells (Sakuragawa et al, 1996, 1997). Further, HAE cells have shown evidence for acetylcholine metabolism, suggesting that they could be applied for intracerebral allografting in neurologic disease in which cholinergic neurons are damaged (Sakuragawa et al, 1997). The same authors have since successfully used HAE as donor cells in a model of brain ischemia, demonstrating that HAE may have therapeutic potential for the treatment of ischemic damage in neuronal disorders (Okawa et al, 2001).

Collectively, these studies have begun to address the question whether amnion can adopt different cell fates in an ectopic environment.

Differentiation of Rat Amnion into Epidermis

The differentiation potential of rat amnion was explored in a study in which the authors sought to determine the lability of amnion by placing it into three ectopic body sites: 1) the kidney capsule; 2) subcutaneous on the back; 3) wrapped in omentum, and also in an in vitro culture model (Knezevic, 1996). In this study, the amnion was not placed on an apposing source of mesenchyme, but instead was transferred or cultured by itself, thus, there was no specific 'message' instructing the amnion to adopt a particular cell fate. **Interestingly, it was discovered that in each of the three body sites as well as the culture model, the amnion had spontaneously differentiated into skin, and in some cases had formed skin appendages such as hair follicles and sebaceous glands.** The formation of hair follicles also suggests that **a new population of stem cells** had been sequestered simultaneously with appendage formation. The authors suggested that the **differentiation of both amniotic ectoderm and embryonic surface ectoderm into skin appear to be morphologically related phenomena,** perhaps via a common developmental pathway (Knezevic 1996).

Cornea Transplantation

Cornea transplantation is the oldest, most common, by far the most successful form of tissue transplantation (Niederkom, 1999). In the United States alone, over 40,000 corneal transplantations are performed each year. Remarkably, however, less than 10% of uncomplicated, first-time cornea transplants will undergo immune rejection even though HLA matching is not routinely performed and the use of immunosuppressive drugs is limited to

only topical corticosteroids. The success of corneal transplantation predates the use of corticosteroids, and thus **further underscores the remarkable immune privilege of corneal allografts.** The explanation for the immune privilege of corneal allografts is based on the obvious avascularity of the cornea, which is believed to somehow sequester the graft from the induction of allodestructive immune responses (Niederkom, 1999). The success of this therapy is dependent on the gradual replacement of the donor's corneal epithelium by the recipient's healthy limbal (stem) cells, and the persistence of donor cells has not been widely studied.

Another group of disorders, the ocular surface diseases, is characterized by the depletion of the stem cell population from the corneal limbus. Thus, conventional cornea transplantation is not successful in these patients since the donor cornea is unable to be repopulated by the recipient's limbal cells. It has recently been shown that sufficient stem cells can be derived from a small 2 mm limbal biopsy and expanded ex vivo by 100 fold ($1\text{-}2x10^7$ cells) to create a graft that is easily transplantable and biologically mimics the corneal surface (Schwab et al, 2001). The small biopsy from the (autologous or allogeneic) donor eye is not sufficient to cause long term damage and does not put the eye at risk. The authors noted that as the limbal cells were cultured, 2-9% remained as stem cells through the cultivation process (Schwab et al, 2001). The survival of expanded limbal grafts suggests that both the limbus and the central cornea were regenerated, indicating that limbal cells were resequestered as stem cells when placed back into the potentiating microenvironment.

Reprogramming of Rabbit Cornea into Epidermis

Recently, the first evidence for reprogramming of corneal epithelium into skin was reported using recombinant models of rabbit cornea and mouse embryonic dermis (Ferraris

et al, 2000). The authors of this study sought to demonstrate the plasticity of corneal epithelium in response to a new mesenchymal signal. Importantly, the authors used central cornea (transit amplifying, or committed cells) rather than limbal (stem) cells to prove that central cornea could be reprogrammed by the dermis and switch from a cornea-specific keratin gene expression profile (K3/12) to a skin-specific keratin pattern (K5/14, K1/12). Furthermore, the reprogrammed cornea even produced hair follicles, pilosebaceous units and sweat glands. These remarkable results showed for the first time that a even differentiated epithelium could be reprogrammed, and suggested that **a new population of stem cells were sequestered in the newly formed appendages.**

Clinical and Military Significance

Based on these lines of evidence, we tested the hypothesis that **human cornea and amnion can be reprogrammed to become skin** and serve as a **biomaterial for gene therapy of human skin diseases and chemical injuries.**

Why would such a material be superior to conventional skin grafting for the treatment of genetic skin diseases?

First, autologous skin grafting on a patient with a genetic disease does not represent an improvement, since the donor (self) cells are also genetically deficient. Therefore, allografts could be used, however, historically these have been shown to be efficiently rejected by recipients. Artificial skin equivalents could be used, however, these provide only a costly and temporary wound cover, and in normal individuals, their own keratinocytes simply use these dressings as a scaffold for re-epithelialization. However, in genetically deficient patients, such as **junctional EB patients** with BPAG2 mutations, wound healing

and cell migration are among the major cellular defects, resulting in eventual loss of the skin equivalent.

In the acute treatment of burns in the military setting, there is little time to expand keratinocyte cultures and perform surgical skin grafting, though in extreme cases, this method is still utilized. **A modality which provided a 'living' bandage that was not rejected would represent a significant advance.**

Most importantly, none of these modalities offers **a means for repopulating the stem cell compartment of the skin**, and by definition then, eventually all donor cells, whether genetically correct or not, would be lost.

The **major advantages of reprogrammed skin** are the following:

1) The supply of **donor amnion** is limitless and inexpensive. Given the lack of immune response to the donor amnion in any of the studies mentioned above, it is likely that amnion or culture HAE cells will represent ideal donor tissues for reprogramming.

2) The supply of **donor cornea** and the techniques for limbal stem cell culture and manipulation are well-described, and the tissue is readily accessible. To avoid immune rejection, donor limbal cells could be harvested from first-degree relatives of JEB patients who are only carriers of BPAG2 mutations, expanded and reprogrammed into skin.

3) A major advantage of using **autologous donor cells** is that, by definition, they are **genetically correct**, insofar as they do not have the same mutation(s) as the recipient. **Thus, one of the two major hurdles of conventional gene therapy approaches is**

overcome by this technique. The wild-type BPAG2 gene exists in the donor genome in the proper transcriptional context and should not be subject to inactivation of expression.

4) Finally, the second major obstacle in gene therapy for skin diseases is the lack of ability to identify a stem cell. **The use of reprogrammed skin overcomes this hurdle as well,** since by definition, the induction of new hair follicles, pilosebaceous units and sweat glands would result in the partitioning of **a new compartment of epidermal stem cells** in the newly formed appendages.

We will continue now to perfect the techniques of both *in vitro* and *in vivo* reprogramming of amnion and cornea into skin. **Once we have established that there is no immune response in animal models, our future goal is to test the use of reprogrammed skin in a limited number of human subjects.**

Now that we have demonstrated feasibility in animal models, it is our goal that this work will be expanded into a study aimed at establishing the utility of reprogrammed skin in the treatment of human genetic skin diseases, such as the different forms of EB, ichthyoses and epidermolytic hyperkeratosis, among others, as well as chemical injuries, chronic wounds and acute burns and injury. These disorders are currently the topic of much gene therapy research, however, largely by the conventional (and not yet successful) approaches of *ex vivo* cell culture, gene replacement, and re-grafting. Further, if these studies prove fruitful, we would also seek funding and approval to begin to test this approach in patients with chemical or other types of burns.

The success of this project would provide a foundation for the clinical application of reprogrammed skin in the treatment of acquired, environmental and genetic skin diseases.

5. Body

Progress in Task 3: In Vitro Model for Reprogramming of Epithelium

As an extension of our work during years 1 and 2, we have developed a newly described modified *in vitro* skin equivalent model (Szabowski et al, 2000; Maas-Szabowski et al, 2001) using embryonic mouse dermis as the inductive source. **Using this model, we have demonstrated that we can induce skin-specific keratin gene expression from amnion after two weeks in culture (Figure 1).** We first developed both an *in vitro* and an *in vivo* model for epithelial reprogramming, using recombinations (dermis+epithelium) with 14.5-day embryonic mouse dermis as the inductive source. These methods build upon the techniques reported by Ferarris et al, however, we have extended our studies to include both amnion and cornea as the donor epithelium. Recombinants of 14.5-day mouse embryonic dorsal dermis combined with adult mouse central cornea and/or amniotic epithelium were cultured at 37°C in tissue culture incubators on soft agar for days 1 and 2 after recombination. This *in vitro* model gives a representation of the earliest changes occurring as the epithelium is being reprogrammed.

In addition to the *in vitro* model, tissue recombinations are also implanted under the kidney capsule of recipient mice. Here, they are allowed to develop in a protected manner for 14 days. All tissue recombinations were examined by H & E staining and immunohistologically at day 1, 2, 7, and 14, with cornea and amnion-specific keratins and skin-specific keratin antibodies. In day 1-2 recombinations, all the cells of the basal layer express the differentiation specific keratins for cornea and amnion, as shown by K12 labelling in corneal epithelium and K8 labelling in amnion epithelium immediately (Fig. 1). It is not surprising to find the keratins of the donor tissue type still expressed at these early time points.

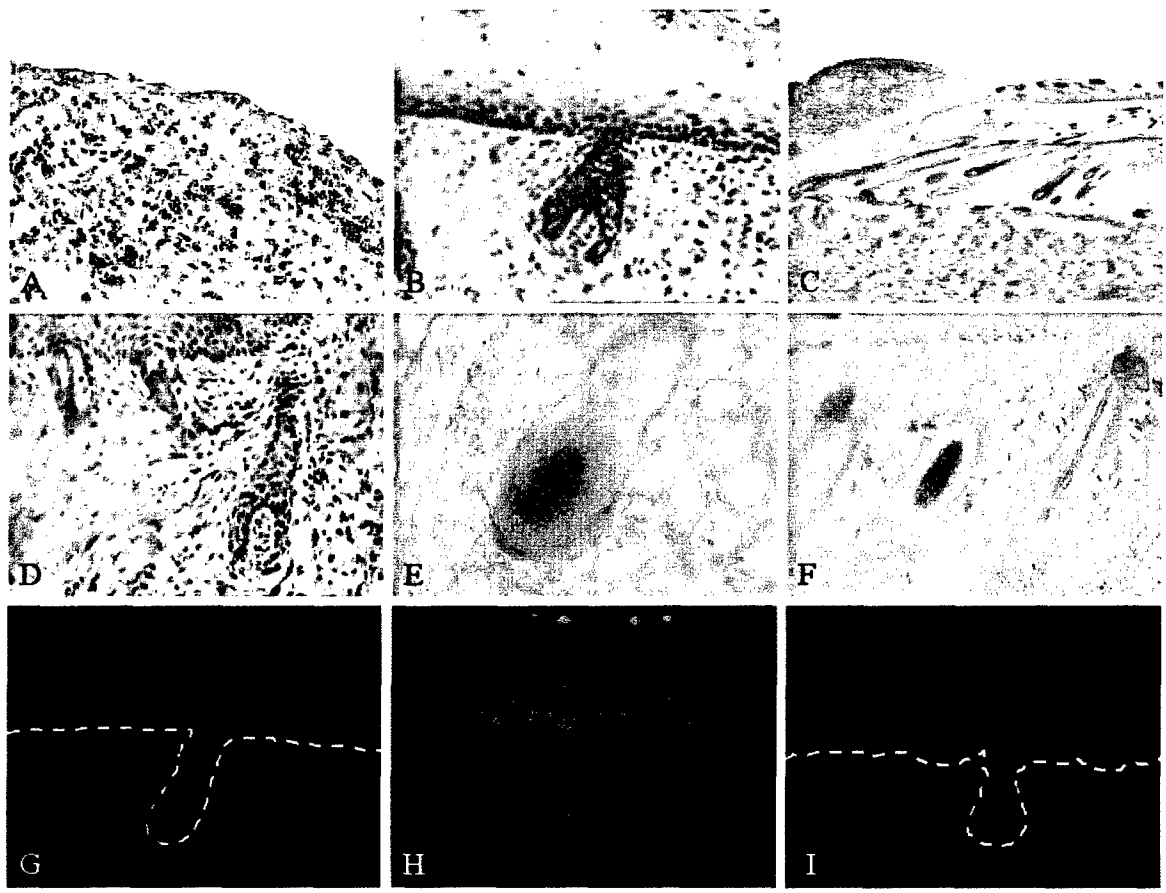

Figure 1. Formation of a new skin at day 2 in incubator, and day 7, 14 after grafting recombinants of adult mouse central corneal epithelium and 14.5-day embryonic mouse dorsal dermis under the kidney capsule. (A) Initially the corneal epithelial cell proliferation is comprised of 2-3 cell layers at day 2 in incubator. (B) At day 7 under kidney capsule, the new corneal epithelium is comprised 6-7 cell layers. Note that the pluristratified epithelium now includes a granular layer and a cornified layer, which characterizes it as epidermal type. (C, D) 14 days after grafting, The new mouse basal layer has formed hair follicle buds in association with mouse dermal cell condensations, which will develop into the dermal papilla. (E) Longitudinal section showing a hair follicle dermal papillae. (F) Longitudinal section showing a hair follicle (h) with associated sebaceous gland. (G) As early as 7 days after grafting, K12 labelling is only present in the suprabasal layers of recombinants. (H) As early as 7 days after grafting, recombinants have formed a new basal layer. (I) After 14 days grafting, the upper epithelial layers shows that the cells containing keratin K12 (red) are shedding. (A-D) H&E staining. (E) Alkaline phosphatase (F) Oil red O (G, I) Immunofluorescent staining with K12 antibody. (H) Hoechst staining.

After 7 days of implantation under the kidney capsule, in heterospecific recombinants of adult mouse corneal epithelium and embryonic mouse dermis from the dorsal (14.5-day embryo) regions, the central cornea/amnion epithelium has become a fully differentiated non-keratinizing epithelium consisting of 6 to 7 layers. A new basal layer was formed, in which the cells no longer express K12 at high levels (Figure 1). K12 labelling is only present in the suprabasal layers, giving the appearance that it is disappearing from the basal layer upwards. Hoechst staining clearly defined these early follicle stages as being derived from the corneal epithelium, and the dermal cells condensing underneath (the dermal papilla precursors) as mouse-derived cells. Grafts also displayed early hair bulbs growing down into the dermis.

At 14 days, fully formed pilosebaceous units are present in the recombinants of mouse dorsal dermis combined with mouse corneal/amnion epithelium. The new mouse basal layer has formed hair follicle buds (hb) in association with mouse dermal cell condensations, which will develop into the dermal papillae (dp). By this stage, staining with the corneal-type (K12) antibody revealed only patchy labelling and in the highest and shedding layers of the epithelium (Figure 2), while most of the lower epithelium is now stained positive with the epidermal-type K5, K1/K10 antibody. On H & E histological analysis, the cornea/amnion epithelium appeared to be transformed into an epidermis characterized by a granular and a cornified layer, associated with several pilosebaceous units. Further, alkaline phosphatase staining for dermal papilla and oil red-O for sebaceous glands are also highly positive.

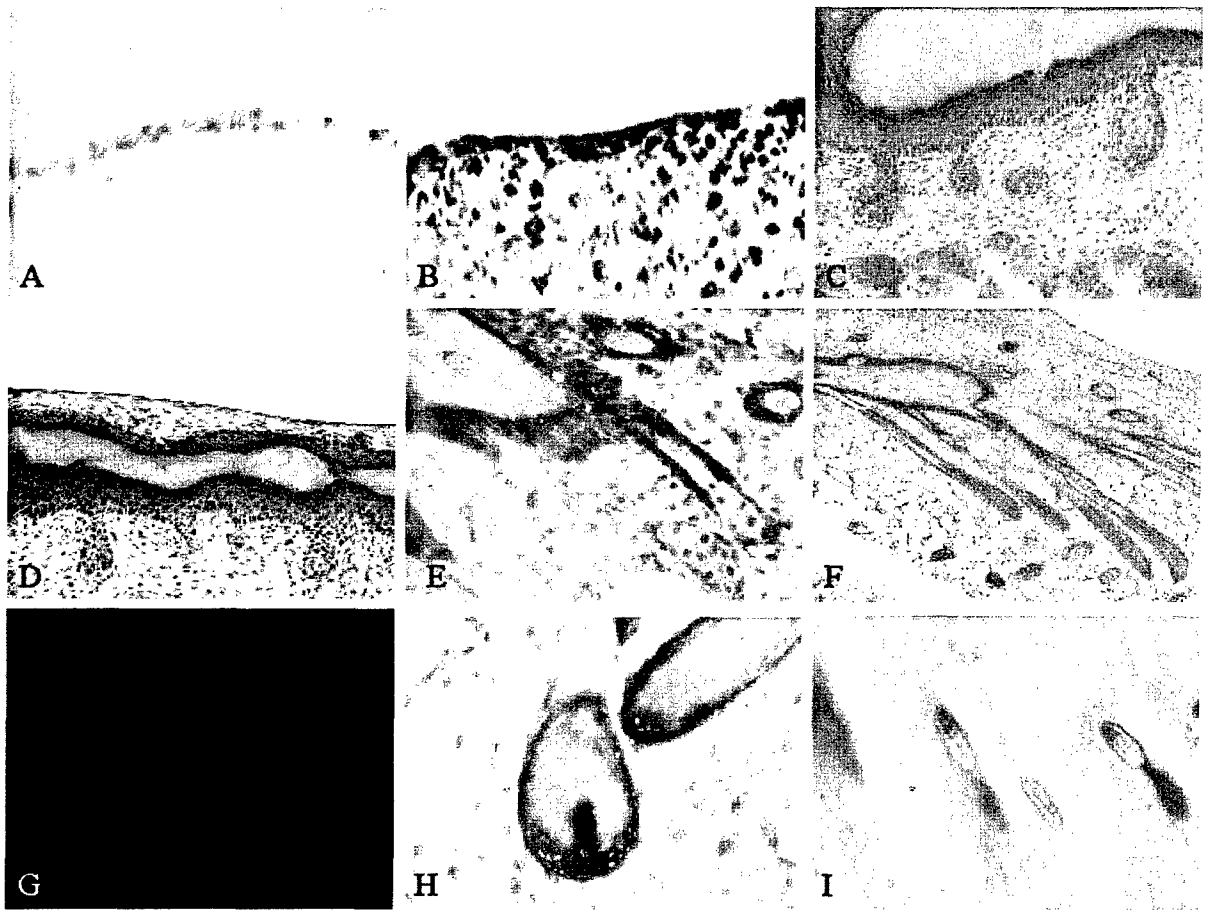

Figure 2. Formation of a new skin at day 2 in incubator, and day 7, 14 after grafting recombinants of adult mouse amnion epithelium and 14.5-day embryonic mouse dorsal dermis under the kidney capsule. (A) the amnion epithelial cell alone. (B) At day 2 in incubator, initially the new amnion epithelium (e) is comprised of 2-3 cell layers. (C, D) At day 7 under kidney capsule, the new amnionic epithelium is comprised of 6-7 cell layers. Note that the pluristratified epithelium now includes a granular layer. (E, F) 14 days after grafting, The new mouse basal layer has formed hair follicle buds in association with mouse dermal cell condensations, which will develop into the dermal papilla. (G) After 14 days grafting, continuous layers of keratin K5 synthesizing cells has formed. (H) Longitudinal section showing a hair follicle dermal papillae. (I) Longitudinal section showing a hair follicle with sebaceous gland. (A-F) H&E staining. (G) Immunofluorescent staining with K5 antibody. (H) Alkaline phosphatase (I) Oil red O.

Our heterospecific recombination experiments showed clearly that signals from day 14.5 embryonic mouse dermis can be recognized by, and the elicit transformation of, adult mouse epithelium to epidermis, hair follicles and sebaceous glands.

In conclusion, our results show that even in the adult, central cornea/amnion epithelial cells retain the surprising ability to transform into an epidermis and to produce appendages such as hair follicles with associated sebaceous glands when recombined with 14.5 days embryonic mouse dermis. These findings support the feasibility of using reprogrammed epithelia for the creating of cell-based bandages for the treatment of chemical wounds and genetic skin diseases, as well as other wound-healing applications.

a. Rationale and Experimental Design

We were focused upon the refinement of the model developed in the initial years of this project. In particular, we will use the model to determine of the pattern and chronology of gene expression changes corresponding to the reprogramming of amnion or cornea into epidermis. We will ask whether the disappearance of the characteristic amniotic keratin (K8) pattern or corneal specific keratin pattern (K3/12) occurs before, after, or at the same time as the appearance of skin specific keratins (K5/14 and K1/10). We will begin to develop the techniques to induce epidermal reprogramming in vitro, in the absence of the inductive day 14.5 dermis. In order for epithelial reprogramming to become clinically relevant, we must determine the molecular and cellular paramaters to recreate the process in vitro. Importantly, we will lift the grafts to the air-liquid interface to determine whether we can induce terminal differentiation, and look for the appearance of markers such as loricrin and involucrin. We will also study the appearance of K19 as an

indication of newly sequestered stem cells. We will initiate these studies using mouse embryonic fibroblasts as the source of inductive mesenchyme, and explore the use of other inductive types of dermal cells, such as hair follicle dermal papilla cells,to recapitulate the molecular signals active in day 14.5 dermis.

For preparation of mouse embryonic fibroblasts, a pregnant female mouse (14.5 days pc) will be sacrificed with CO_2. 4-6 embryos are removed and washed in PBS. Liver, intestine, head and limbs are removed. After washing in PBS embryos are minced with scissors in trypsin/EDTA until it can be taken up in a 10 ml pipette. The mixture is pipetted up and down several times and incubated at 37°C for 10 minutes in a Petri dish. Pipetting with 5 ml pipette is followed by another 10 minutes of incubation. Then, all contents are transferred into a 50 ml tube. The cellular debris is allowed to settle out over a period of 2 minutes. The supernatant is removed into a fresh tube, mixed with MEF media (up to 50 ml) and centrifuged at 1000 RPM for 5 min at 10°C. The pellet is re-suspended in MEF medium and cells are plated in 250 ml flasks. The next day the medium is changed to remove cellular debris and cells are incubated for another 24 h.

Rat tail collagen (Sigma #C7661) is dissolved in 12 mM HCL (4 mg/ml) and incubated at 4°C overnight for swelling. Then, one part of 10x Hank's balanced salt solution (Gibco #14180-061) is added to 8 parts collagen solution and pH set to 7.0 (on ice). The collagen is immediately mixed with one part MEF (mouse embryonic fibroblasts; 500,000 cells in 2 mlcollagen) in 'skin model medium' (SMM). 2 ml of the final mixture is immediately poured in cell culture inserts placed previously in 6-well culture plates (Falcon, Multiwell tissue culture plate, # 3846) filled with 2 ml of SMM. After 1 h polymerization (at 37°C), SMM (2 ml) is added on the top of the gel.

Pieces of mouse amnion will be placed on top of polymerized collagen (epithelial side up) and weighed down using sterile teflon rings. The system is incubated undisturbed for two days. After that, the medium was removed from inside of the ring while it was kept outside the ring. Incubation was continued for 14 days. Central corneas and limbus cells will be harvested from adult mice, and either cultured or placed directly in the same in vitro model described. Immunohistochemistry will be performed every 2-3 days for 14-21 days using antibodies for K3/12, K8, K1/10, K5/14, loricrin, involucrin and K19 either from commercial sources (Sigma, Boehringer, Neo Markers) or through collaboration.

b. Outcomes, Expected Results and Alternatives

While it is anticipated that lifting the in vitro recombinant grafts to the air interface will induce differentiation, it is possible that reprogrammed amnion or cornea may not respond in the same manner as keratinocytes. If we encounter this dilemma, we will introduce compounds into the culture medium which have been shown by others to induce differentiation and barrier formation in fetal rats (Hanley et al, 1999; Billoni et al, 2000). These compounds include activators of the nuclear hormone receptor PPAR, in particular, clofibrate (1 mg) and linoleic acid (1 mg). Both agents have been shown to promote epidermal maturation, barrier formation and stratum corneum development, suggesting that they may be of use in promoting differentiation should this prove to be a challenge in our model.

We will begin these experiments using day 14.5 embryonic mouse dermis as the inductive source, since these cells were shown to be sufficient to reprogram rabbit cornea in the kidney capsule model (Ferraris et al, 2000). Although unexpected, should these cells prove insufficient for induction of reprogramming in our model, we will add or substitute cultured rodent dermal papilla and/or dermal

sheath cells as the inductive mesenchyme. Other experiments in the lab are ongoing to identify the nature of the day 14.5 inductive mesenchymally-derived signal using microarray analysis and the use of dermal papilla-cultured conditioned media. This information could nicely synergize with our proposed experiments and add an additional experimental alternatives to increase the likelihood of inducing epithelial reprogramming in the invitro setting. Dr. Colin Jahoda is a long-standing expert in the field of epithelial recombinations and induction and will provide us advice on the donor tissues as well as specialized dermal cells as needed.

***Continuation of Task 3: In vivo* model of epithelial reprogramming**

We will then test whether we can recapitulate the *in vitro* results of Specific Aim 1 in an animal model of wounding. We will test this approach using an *in vivo* model first with immunodeficient mice, and finally with wild-type recipients to determine whether the grafts can be induced *in vivo*. Importantly, we will learn whether any rejection occurs between unmatched donors and recipients. Our experimental plan involves the following strategies: 1) C57BL/6 mouse amnion grafted onto SCID (Charles River C.B.-17/scid) mice; 2) C57BL/6 mouse amnion grafted onto C57BL/6 mice (immunologically compatible autograft); 3) BALB/c cornea/limbus grafted onto C57/BL6 mice (mismatched allograft); 4) cultured C57BL/6 mouse cornea/limbus grafted onto SCID mice; 5) cultured C57BL/6 mouse cornea/limbus grafted onto C57BL/6 mice (immunologically compatible autograft); 6) cultured BALB/c cornea/limbus grafted onto C57/BL6 mice (mismatched allograft). Grafts will be analyzed for microscopic and macroscopic criteria (below), and the changes in gene expression defined in above, as well as for any signs of immune response or rejection.

a. Rationale and Experimental Design

We will introduce 2x2 cm full-thickness wounds in 15 mice in each of the five groups by surgically excising the skin. Under aseptic conditions, the skin will be excised to the level of the muscle with careful hemostasis. Wound margins will be tatooed with India ink, allowing for photometric and visual standardization and sequential photographs. The wound area will be covered with fresh amnion or cultured cornea cells or left open as a control wound. The graft will be sutured to the wound margin and covered with a nonadherent semi-occlusive gauze dressing. When needed, animals will have wound chambers to protect the wounds. Wounds will be evaluated macroscopically and microscopically after 2, 4, 5, 10 and 14 days, three different mice per examination for each type of wound. The primary adherence or 'take' of the graft will be assessed at 4 and 10 days by gross inspection and histology.

All grafts will be scored based on three macroscopic (adherence, color, pliability) and three microscopic (structural integrity, leukocyte infiltration, adherence) criteria. The Kruskal-Wallis test will be used to statistically compare the total performance scores of the different groups. The Mann-Whitney test will be used to compare one group to another when the difference among groups is found to be significant (Dawson-Saunders and Trapp, 1990). In addition to these tests, immunohistochemistry will also be performed with all markers specified in Aim 1, as well as careful assessment for immunological markers if necessary. Here we will look for evidence of stratification, expression of skin specific keratins, terminal differentiation markers, as well as immunological evidence of rejection versus tolerance.

We predict that skin appendages such as pilosebaceous units will be present in the reprogrammed skin on the basis of previous studies (Ferraris et al, 2000). To help us

evaluate this possibility, we have enlisted the expert help of Dr. Colin Jahoda, University of Durham, UK, who conducted the rabbit cornea experiments referenced above.

b. Outcomes, Expected Results and Alternatives

We anticipate that there may be some infiltration of the grafts by surrounding keratinocytes during normal wound healing. Therefore, to prevent this from occurring and to allow the transplanted cornea or amnion the maximal opportunity to adhere and 'take', we will introduce the use of a wound chamber embedded subcutaneously to prevent inward migration of keratinocytes. We will use either a modified chamber (P.A. Medical, Columbia, TN) which consists of a flexible vinyl or silicon ring bonded to an adhesive base. We will attach the adhesive ring to a flat base which will be imlanted subcutaneously and form a physical barrier, preventing the migration of keratinocytes into the graft. A similar modified system was recently reported by Mizoguchi et al. (Mizoguchi et al, 2001) for use in implanting human skin equivalents onto the back of nude mice.

While the initial wound coverage will be nonadherent gauze dressing, clearly there can be significant differences in wound healing depending upon the choice of dressing. Should the need arise and we encounter difficulties, we will also attempt different dressings including polyurethane foam (Allevyn), paraffin gauze, polythene sheet (Opsite) and silicone sheets if necessary. In addition, the timing of exposing the wounds to the air versus allowing for wound dessication will also be taken into account when developing the protocol for wound coverage which best promotes epithelial reprogramming.

Given the known challenges in culturing mouse keratinocytes, we are aware that there may be technical challenges involved in establishing culture conditions from corneal cells as well. Should we encounter difficulty in the culture or propagation of mouse corneal cells, or the wounds be of insufficient size, we will convert the experiments to be carried out in the brown Norway as wild type and Charles River immunodeficient rats (Crl:Rnu BR (Nude) as recipients. Our neighbor, Dr. Rebecca Morris is an expert in mouse keratinocyte culture and is available to assist us in this work.

6. Key research accomplishments

In this final year of funding, we have greatly expanded the scope of the initial skin equivalent experiments proposed, to include the use of alternative epithelia such as cornea and amnion as the source of reprogrammed keratinocyte precursors. We have been working extensively on the *in vitro* skin model described in the proposal initially. We first proceeded with the assembly of a model consisting of normal cells, to be used as a control during the recombination experiments. According to the experimental outline, we performed a two-step assembly in all cases. During the first phase, we developed a multi-layer fibroblast base, which served as a recipient surface for the keratinocytes, amnion or cornea in the second phase. After successful attachment of the seeded epithelial cells, the system was elevated to the air-liquid interface to allow the multi-layer growth and differentiation of the epithelium.

We built the skin models using different combinations of starting materials. All culture systems were initially based on a collagen matrix, which contained a variable proportion of fibroblasts. Their attachment, cell division and growth characteristics were observed and evaluated. The human cell based models were compared side-

by-side with skin models utilizing cell types of murine origin. In the in vivo experiments, we will use inductive dermis as the reprogramming source and these experiments will be expanded as outlined in the continuing experimental plan.

7. Reportable Outcomes

1. Preliminary data from these studies served as the basis for an **NIH R21 grant that was recently funded** focused on epithelial reprogramming as an alternative gene therapy approach.

2. We have recently **obtained a grant from the Steven and Michelle Kirsch Foundation** to continue working on the mechanisms underlying epithelial reprogramming of skin.

3. In October 2003, we applied for a **Dermatology Foundation Research Career Development Award** for Dr. Kai Sun to continue this very important work.

4. In May 2003, this work was presented at the **International Investigative Dermatology Meeting** in Miami Florida, in an **Oral Talk** entitled: *"Epithelial Reprogramming of Adult Mouse Central Cornea to Hair Follicle and Sebaceous Glands Under Inductive Dermal Influences: A Novel Approach to Cellular Therapy"*. Authors: Sun, K., Jahoda, C.A.B. and Christiano, A.M. J. Invest. Dermatol.

8. Conclusions

Upon successful completion of this project, we have acquired sufficient preliminary data in model systems with which to justify a trial of reprogrammed skin in patients with genetic skin disorders in the future. While this study has evolved rapidly, keeping pace with the stem cell field around it, we have never lost sight of our **original stated goals of developing a cellular therapy for EB and for chemical burns.** At the outset, we could have never predicted the speed with which the gene therapy and tissue engineering fields have advanced. We have done our very best to be sure that the goals of the US Army and Medical Research and Materiel Command were not only met, but we believe, exceeded.

Finally, we are extremely proud to report that this recent work focused on epithelial reprogramming as an alternative gene therapy approach **was recently funded (on its first review) by the NIH as an R21 award to continue to advance this very exciting and promising preliminary data.** In addition, we **received a grant from the Kirsch Foundation** to continue this work, and have **applied to the Dermatology Foundation** for funding as well.

Our work was featured prominently at the **International Investigative Dermatology Meeting** in May 2003 in Miami, Florida, where it was invited for presentation as an **Oral Talk** entitled *"Epithelial Reprogramming of Adult Mouse Central Cornea to Hair Follicle and Sebaceous Glands Under Inductive Dermal Influences: A Novel Approach to Cellular Therapy".* Our findings were greeted with an enthusiastic response by many colleagues and investigators from around the world who are also engaged in this field of research.

In closing, we are enourmously grateful for the generous support of the USAMRC during this award period. Particularly in light of the events of September 11, 2001, which has affected us greatly in New York City, we have been deeply honored to serve in this important capacity. Due to your support, our progress has been swift toward the goal of enhancing the military readiness of chemical injuries. Should the USAMRC deem that our work is worthy of continuation by a future funding mechanism, we would once again be proud to continue with this work.

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Okawa H, Okuda O, Arai H, Sakuragawa N, Sato K (2001) Amniotic epithelial cells transform into neuron-like cells in the ischemic brain. Neuroreport 12: 4003-7

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10. Appendices

1. Abstract, International Investigative Dermatology Meeting
2. EB Chapter
3. Curriculum Vita

also specifically expressed in the epidermis and other stratified epithelia. A comparison of mouse and human genomic sequences confirmed that the region on human chromosome 10, which is syntenic with mouse chromosome 13, encodes human CLSP, as well as the calmodulin-like protein (CLP/CALML3). Future studies, including conditional knockout mouse models, will further elucidate the function of these calmodulin-like proteins in skin development and differentiation.

0561

Epithelial reprogramming of adult mouse central cornea to hair follicle and sebaceous glands under inductive dermal influences: a novel approach to cellular therapy

K Sun,[1] C Jahoda[2] and AM Christiano[1] *1 Derm and Genet & Dev, Columbia U, NY, NY and 2 Biol Sci, U Durham, Durham, United Kingdom*

Gene therapy in the skin has been hampered by the inability to target/identify a stem cell, and the lack of sustained gene expression. Several lines of evidence about the plasticity of stem cells in general have prompted us to consider a novel approach to the treatment of inherited skin disorders. Rather than searching for markers of the epidermal stem cell itself, we aimed at reprogramming other epithelia into skin under the appropriate inductive (dermal) influences in vivo. If the donor cells, such as cornea, were taken from an immunologically-compatible individual or did not elicit an immune response, such cells could overcome the two major obstacles in gene therapy approaches: gene introduction and targeting the stem cell. Building on the previous work of Ferarris et al, we performed tissue recombination experiments using embryonic mouse dermis from day 14.5 embryos combined with adult mouse central cornea cells. Recombinant grafts were implanted beneath the kidney capsule of normal mice and retrieved after two weeks. Fully formed pilosebaceous units with mature hair shafts and sebaceous glands had been induced, containing reprogrammed central cornea cells as their epithelial component. Thus, we have demonstrated that differentiated adult cells can regenerate epidermis under the appropriate dermal influences. In this approach to cellular therapy, ectopic donor epithelial cells, by definition, would contain an intact gene-of-interest, and importantly, it is predicted that epidermal stem cells would be sequestered during the induction of the new skin and hair follicle, thus providing a lifelong supply of genetically corrected cells. We are currently expanding this technology to include other sources of donor epithelium, as well as developing methodology to recapitulate the molecular events of epithelial reprogramming in vitro.

0563

Manipulation of retinoid metabolism

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Vitamin A and its derivatives have been shown to provide a variety of skin benefits. Prescription retinoic acid in particular has been shown to repair photodamaged skin and provide improvements to lines/wrinkles and hyperpigmented skin. Cosmetic vitamin A derivatives, such as retinol and retinyl esters, have been reported to provide modest improvement in the appearance of photoaged skin con-

12/1/03

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BOARD CERTIFICATION

Fellow of the American Board of Medical Genetics
Certification in Clinical Molecular Genetics
2002-present

EDUCATION

1983-1987	Douglass College Rutgers University New Brunswick, New Jersey B.A. in Biology
1987-1990	Rutgers University Graduate School UMDNJ - Graduate School of Biomedical Sciences Joint Graduate Program in Microbiology New Brunswick, New Jersey M.S. in Microbiology & Molecular Genetics
1987-1991	Rutgers University Graduate School UMDNJ - Graduate School of Biomedical Sciences Joint Graduate Program in Microbiology New Brunswick, New Jersey Ph.D. in Microbiology & Molecular Genetics

FELLOWSHIPS

1991-1992	Post-Doctoral Fellow

Department of Dermatology
Thomas Jefferson University
Jefferson Medical College
Philadelphia, Pennsylvania

1992-1993 Research Instructor
Department of Dermatology
Thomas Jefferson University
Jefferson Medical College
Philadelphia, Pennsylvania

1997-1999 Fellowship in Clinical Molecular Genetics
Division of Clinical Genetics
Department of Pediatrics
Presbyterian Hospital
New York, NY

ACADEMIC APPOINTMENTS

1993-1995 Research Assistant Professor
Department of Dermatology
Thomas Jefferson University
Jefferson Medical College
Philadelphia, Pennsylvania

1993-1995 Adjunct Assistant Professor
Laboratory for Investigative Dermatology
The Rockefeller University
New York, New York

1995-1996 J. Lowry Miller Assistant Professor of Molecular Dermatology (in Dermatology)
Department of Dermatology
College of Physicians and Surgeons
Columbia University
New York, New York

1996-1999 Herbert Irving Assistant Professor of Molecular Dermatology
Department of Dermatology
College of Physicians and Surgeons
Columbia University
New York, New York

1998-1999 Herbert Irving Assistant Professor of Molecular Dermatology and Genetics & Development
Department of Dermatology
College of Physicians and Surgeons
Columbia University
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1999-present Associate Professor of Molecular Dermatology and Genetics & Development

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College of Physicians and Surgeons
Columbia University
New York, New York

2001-present Director of Research
Department of Dermatology
College of Physicians and Surgeons
Columbia University
New York, New York

AWARDS AND HONORS

1983-1987 Douglass College Scholar - Rutgers University
Four Year Full Tuition Merit Scholarship Program

May 1991 Ph.D. Awarded in Microbiology
Rutgers University, New Brunswick, New Jersey
"The Human Tropoelastin Gene: Allelic Heterogeneity, Evolutionary Divergence, Identification of Restriction Fragment Length Polymorphisms and Linkage Analyses in Families with Pseudoxanthoma Elasticum"

December 1993 Emanuele Stablum International Award for Dermatology
Istituto Dermopatico dell'Immacolata, Roma, Italy

May 8, 1996 The Fifth Annual Phillip and Ruth Hettleman Lecturer
St. Luke's/Roosevelt Hospital
New York, New York

July 1996-June 1999 Herbert Irving Clinical Scholar
Columbia-Presbyterian Medical Center

April 2000 Louis Forman Visiting Professor of 2000
St. John's Institute of Dermatology
London, UK

March 6, 2001 New York City Mayor's Award for Excellence in Science and Technology – Young Investigator's Award

May 16, 2001 Doctor Harold and Golden Lamport Research Award for "Excellence In Clinical Sciences", Columbia University, New York, NY

September 2003 CERIES Research Award

EDITORIAL EXPERIENCE

Editor, **Experimental Dermatology**

Munksgaard International Publishers, Ltd.
1996-present.

Consulting Editor, **Journal of Clinical Investigation**
American Society for Clinical Investigation
2002-2003

Associate Editor, **Journal of Clinical Investigation**
American Society for Clinical Investigation
2003-present

ORGANIZATIONAL EXPERIENCE

New York Skin Biology Club
Co-founder and Meeting Co-chairperson
March 2001- present

Society for Investigative Dermatology
Scientific Program Committee
May 2005
Co-Chair

Gordon Conference on Epithelial Differentiation and Keratinization
July 2005
Chair

SOURCES OF FUNDING

PREVIOUS

Grant:	Pre-Doctoral Fellowship
Agency:	American Heart Association
Amount:	
Period:	1990-1991
Role:	Principal Investigator

Grant:	Elastin Gene Mutations in Inherited Skin Disorders
Agency:	Dermatology Foundation Research Fellowship
Amount:	
Period:	1/91-1/92
Role:	Principal Investigator

Grant:	Type VII Collagen Gene Mutations in DDEB
Agency:	Dermatology Foundation Research Fellowship
Amount:	
Period:	1/92-1/93
Role:	Principal Investigator

Grant:	Application of Gene Therapy to the Dystrophic Forms of Epidermolysis Bullosa
Agency:	Dermatology Foundation Career Development Award
Amount:	

Period: 1/93-9/95
Role: Principal Investigator

Grant: Development of a PCR-Based Mutation Detection for the Type VII Collagen Gene: Application to Prenatal Diagnosis of the Dystrophic Forms of Epidermolysis Bullosa
Agency: Dermatology Foundation Research Grant
Amount:
Period: 7/95-7/96
Role: Principal Investigator

Grant: Development of DNA-Based Prenatal Diagnosis and Mutational Analysis in the Junctional and Dystrophic Forms of Epidermolysis Bullosa
Agency: March of Dimes Birth Defects Foundation
Basil O'Connor Starter Scholar Research Award
Amount: 0
Period: 2/95-1/97
Role: Principal Investigator

Grant: Gene Therapy for Inherited Skin Disorders
Agency: Columbia-Presbyterian Medical Center
Amount:
Period: 7/96-6/97
Role: Principal Investigator

Grant: Gene Therapy for Recessive Dystrophic Epidermolysis Bullosa
Agency: American Skin Association Research Grant Award
Amount:
Period: 3/96-2/98
Role: Principal Investigator

Grant: Research Grant Award
Agency: National Alopecia Areata Foundation
Amount:
Period: 7/97-6/98
Role: Principal Investigator

Grant: Research Grant Award
Agency: American Porphyria Foundation
Amount:
Period: 7/97-6/98
Role: Principal Investigator

Grant Title: Preimplantation Genetic Diagnosis for Inherited Skin Disorders
Agency: Irving Center for Clinical Research CPMC
Amount:
Period: 7/96-6/99
Role: Principal Investigator

Grant Title: Positional Cloning of Inherited Alopecias
Agency: National Alopecia Areata Foundation
Amount:
Period: 7/98-6/99

Role: Principal Investigator

Grant Title: Gene Therapy for Inherited Skin Disorders
Agency: Johnson & Johnson Foundation
Amount:
Period: 10/97-9/00
Role: Principal Investigator

Grant Title: Structural and Mutational Analysis of the LAMA3 Gene
Agency: NIH/NIAMS R29-AR43602
Amount:
Period: 9/95-8/00
Role: Principal Investigator

Grant Title: Skin Disease Research Center
Agency: NIH-NIAMS Grant Award P30-AR44535
Amount:
Period: 07/97-08/01
Role: Director Core C: Genotyping & Molecular Diagnostics
Director: Core Programs

Grant Title: Molecular Genetics of the Keratodermas
Agency: NIH/NIAMS Grant Award K02-AR02047
Amount:
Period: 1/98-12/02
Role: Principal Investigator

Grant Title: Gene Therapy for Inherited Skin Disorders
Agency: US Army Medical Research
Amount:
Period: 11/99-10/02
Role: Principal Investigator

Grant Title: Cellular Therapy for Hair Follicle Disorders
Agency: The Kirsch Foundation
Amount:
Period: 7/00-6/02
Role: Principal Investigator

ACTIVE

Grant Title: **Molecular Genetics of the Keratodermas**
Agency: NIH/NIAMS Grant Award **R01-AR44924**
Amount:
Period: 7/98-6/03
Role: **Principal Investigator**

Grant Title: **Cellular Therapy for Hair Follicle Disorders**
Agency: The Kirsch Foundation
Amount:
Period: 1/03-12/04
Role: Principal Investigator

Grant Title: **Gene Therapy Model of Dystrophic Epidermolysis Bullosa**
Agency: NIH/NIAMS Grant Award **R01-AR43602-07**
Amount:
Period: 4/01-3/06
Role: Principal Investigator

Grant Title: **Functional Analysis of the Hairless Protein**
Agency: NIH/NIAMS Grant Award **R01-AR47338**
Amount:
Period: 7/1/01-6/30/06
Role: Principal Investigator

INVITED VISITING LECTURESHIPS

January 25, 1994	NIH/NCI Dermatology Branch Bethesda, Maryland
February 2, 1994	University of Pennsylvania School of Dental Medicine Philadelphia, Pennsylvania
March 30, 1994	State University of New York Health Sciences Center at Brooklyn Department of Dermatology Brooklyn, New York
November 9, 1994	State University of New York at Stony Brook Department of Oral Biology and Pathology Stony Brook, New York
April 24, 1995	Howard University Department of Genetics Washington, D.C.
May 5, 1995	Hahnemann University Department of Dermatology Philadelphia, Pennsylvania
December 6, 1995	College of Physicians Philadelphia, PA
December 21, 1995	University of Pennsylvania Department of Dermatology Philadelphia, PA
May 8, 1996	The Fifth Annual Phillip and Ruth Hettleman Lecturer Department of Medicine Grand Rounds St. Luke's/Roosevelt Hospital New York, New York
May 22, 1996	Neonatology Grand Rounds

	Columbia-Presbyterian Medical Center New York, New York
July 16, 1996	New York Hospital/Cornell Medical Center Department of Dermatology Grand Rounds New York, New York
September 10, 1996	Department of Pediatrics, Division of Clinical Genetics Columbia-Presbyterian Medical Center New York, New York
September 13, 1996	Department of Pediatrics Grand Rounds Columbia-Presbyterian Medical Center New York, New York
October 2, 1996	Department of Dermatology Bowman Gray School of Medicine Greensboro, North Carolina
October 16, 1996	Department of Anatomy and Cell Biology Columbia-Presbyterian Medical Center New York, New York
October 23, 1996	Department of Dermatology Lincoln Hospital Bronx, New York
October 25, 1996	Department of Dermatology New York University Medical Center New York, New York
November 5, 1996	Department of Genetics & Development Columbia University New York, New York
November 15, 1996	Department of Dermatology The University of Iowa Hospitals and Clinics Iowa City, Iowa
December 11, 1997	Institute for Human Genetics University of Minnesota Minneapolis, MN
December 12, 1997	Department of Dermatology University of Minnesota Minneapolis, MN
July 24, 1998	Department of Human Genetics Mt. Sinai School of Medicine New York, NY
November 17, 1998	Department of Dermatology University of Pennsylvania Philadelphia, PA

November 19, 1998	Department of Pediatrics Grand Rounds The New York Hospital-Cornell Medical Center New York, New York
December 16, 1998	Department of Dermatology State University of New York Health Science Center Brooklyn, New York
August 19, 1999	Integriderm/Research Genetics Huntsville, AL
November 8-10, 1999	Department of Immunology University Federico II Natples, Italy
December 17, 1999	New York University Department of Dermatology
January 18, 2000	MD Anderson Cancer Center Houston, Texas
April 6, 2001	University of Bologna Bologna, Italy

INVITED SPEAKER

February 5, 1995	American Academy of Dermatology 53rd Annual Meeting Symposium 317: Advances in Biological Science in Relation to Dermatology Topic: "Gene Therapy"
February 5, 1995	American Academy of Dermatology 53rd Annual Meeting Symposium 318: What's New and Hot in Clinical Research? A Tribute to Lawrence E. Shulman, M.D. Topic: "Understanding the Importance of Gene Defects in Skin Disease"
February 5, 1995	American Academy of Dermatology 53rd Annual Meeting Course 111: Molecular Biology for Dermatologists Topic: "Survey of State-of-the-Art Technologies: PCR, cDNA Cloning, Mutation Detection Systems, Transgenic Animals"
February 5, 1995	American Academy of Dermatology 53rd Annual Meeting Course 112: Genetics and Genodermatoses Topic: "Molecular Techniques for Diagnosis and Gene Therapy"
May 19, 1995	American Society for Dermatologic Surgery 22nd Annual Meeting In-Depth Symposium 501: Biology of Aging and Photoaging

	Joint Session of the Society for Investigative Dermatology and the American Society for Dermatologic Surgery Topic: "Structure and Function of Aged and Photoaged Dermis"
November 11, 1995	Dystrophic Epidermolysis Bullosa Research Association Research Conference Topic: "Prenatal Diagnosis and Reclassification of Junctional and Dystrophic EB"
February 11, 1996	American Academy of Dermatology 54th Annual Meeting Course 113: Application of Molecular Techniques in Dermatology Practice Topic: "Prenatal Testing for Heritable Skin Diseases"
April 30, 1996	International Symposium on Epidermolysis Bullosa "Dystrophic EB and Mutations in Type VII Collagen"
April 30, 1996	International Symposium on Epidermolysis Bullosa "Impact of DNA Diagnostics on EB: Molecular Reclassification and Prenatal Diagnosis"
May 4, 1996	AFCR/SID Biomedicine1996 "Clinical Implications of Basic Research in EB"
May 5, 1996	SID Session Chair Introduction "Mutations as Determinants of Clinical Phenotypes"
May 30, 1996	Clinical Dermatology 2000 Symposium: Genetics and the Skin "Photosensitivity Associated with Genetic Diseases"
May 31, 1996	Clinical Dermatology 2000 Symposium Photosensitivity 1990-2010 "Prenatal Diagnosis of Heritable Skin Diseases"
June 12, 1996	Basement Membrane Gordon Conference "Molecular Complexity of the Cutaneous Basement Membrane: Lessons from Epidermolysis Bullosa"
October 17, 1996	New York Human Genetics Meeting "Vohwinkel's Keratoderma and the Epidermal Differentiation Complex on 1q21"
November 13, 1996	Society for the Advancement of Women's Health Research Sixth Annual Scientific Advisory Meeting Genetics and Women's Health "Impact of Genetics on Dermatology" Representing the Joint Committee for the Advancement of the Dermatologic Health of Women
November 14, 1996	Howard Hughes Medical Institute Fall Symposium on Cell-Extracellular Matrix Interactions in

	Development and Disease "Molecular Complexity of the Cutaneous Basement Membrane: Lessons from Epidermolysis Bullosa" The University of Iowa College of Medicine Iowa City, IA
February 25, 1997	Ortho Pharmaceuticals Raritan, New Jersey
March 23, 1997	American Academy of Dermatology 55th Annual Meeting San Francisco, CA
June 20, 1997	19th World Congress of Dermatology Sydney, Australia "Gene Defects in Skin Diseases"
July 16, 1997	FibroGen, Inc. South San Francisco, CA
August 2, 1997	American Academy of Dermatology Summer Meeting New York, New York Course 501 The Basic Science of Dermatology "Applications of Molecular Biology in Dermatology"
August 9, 1997	Epidermolysis Bullosa Patient Conference "Prenatal and Preimplantation Diagnosis" Chapel Hill, North Carolina
September 27, 1997	New England Dermatological Society Case Commentator Yale University School of Medicine New Haven, Connecticut
October 14, 1997	8th International Symposium on Basement Membranes "Basement Membrane Diseases of the Skin" Cutaneous Biology Research Center Charlestown, Massachusetts
May 4-5, 1998	Hereditary and Acquired Bullous Dermatoses Satellite Meeting of International Investigative Dermatology Salzburg, Austria "DNA-Based Prenatal Diagnosis and Preimplantation Diagnosis"
June 8-11, 1998	International Symposium on Prenatal Diagnosis "Prenatal Diagnosis of Inherited Skin Disorders" Los Angeles, CA
June 21-24, 1998	Society for the Advancement of Women's Health Research Washington, DC Session Chair: "Advances in Dermatological Diseases"

September 17, 1998	International Society of Hair Restoration Surgery Washington, DC "Molecular Basis of Inherited Alopecia"
October 6, 1998	Encino-Tarzana Medical Center Medicine 2000 Distinguished Lecture Series "Molecular Basis of Inherited Hair Loss" Sherman Oaks, California
October 16, 1998	Missouri Dermatological Society Hair and Nail Symposium "Molecular Basis of Inherited Hair Loss" St. Louis, MO
October 24-26, 1998	Symposium on Genodermatoses Institute for Human Genetics University of Minnesota "Molecular Basis of Keratodermas" "Prenatal Diagnosis in Inherited Skin Disorders" Minneapolis, MN
October 28, 1998	American Academy of Dermatology 1998 Dermatology Update "Gene Mapping" New York, NY
November 5, 1998	Third International Research Workshop on Alopecia Areata Washington, DC "Cloning of the Hairless Gene in Mouse and Humans"
January 15, 1999	Philadelphia Dermatological Society "Molecular Basis of Inherited Alopecias" Philadelphia, PA
March 3, 1999	Hair Restoration Surgery Meeting "Molecular Basis of Inherited Alopecias" Orlando, FL
March 19, 1999	American Academy of Dermatology 56th Annual Meeting Course: Clinical Disease and its Molecular Basis Topic: "Molecular Basis of Inherited Blistering Disorders" New Orleans, LA
March 20, 1999	American Academy of Dermatology 56th Annual Meeting Course:Advanced Pediatric Dermatology Symposium Topic: "Prenatal and Preimplantation Diagnosis of Inherited Skin Disorders" New Orleans, LA
May 2-4, 1999	Second Annual Global Convocation on Hair Restoration Surgery "Molecular Basis of Inherited Hair Loss" Rancho Mirage, CA

June 13-17, 1999	Society for Developmental Biology "Molecular Basis of Inherited Alopecias" Charlottesville, VA
June 23-25, 1999	Symposium for Dr. Albert Kligman Department of Pharmacology Rutgers University Piscataway, NJ
July 18-23, 1999	Gordon Conference on Epithelial Differentiation and Keratinization Session chair and Speaker: "Hair, nails and teeth" Tilton School New London, NH
July 26-29, 1999	Gordon Conference on Collagen "Collagen Gene Mutations in Diseases of the Skin" Colby-Sawyer School New London, NH
July 30, 1999	American Academy of Dermatology Summer Meeting "Applications of Molecular Biology to Dermatology" New York, NY
October 22, 1999	Dermatology Update for the Milleniun "The Search for Hair Loss Genes" San Francisco, CA
March 17, 2000	American Academy of Dermatology "Molecular Basis of Inherited Hair Loss" San Francisco, CA
April 7, 2000	Karolinska Hospital Dermatology Meeting Stockholm, Sweden
April 10-11, 2000	Louis Forman Visiting Professor 2000 St. John's Institute of Dermatology London, UK
June 14, 2000	Israel Society of Dermatology and Venerology 24th Annual Meeting Jerusalem, Israel
June 24, 2000	National Alopecia Areata Foundation International Patient Conference Norfolk, VA
July 6, 2000	Sociedad de Dermatologia de Nuevo Leon Monterrey, Mexico
August 4, 2000	American Academy of Dermatology Summer Meeting Nashville, TN

September 24-26, 2000	EB 2000 London, UK
October 3-5, 2000	The Jackson Laboratories Mouse and Human Genomics Meeting Bar Harbor, Maine
December 13, 2000	OSI Pharmaceuticals Uniondale, NY
March 31, 2001	American Society of Investigative Pathology Symposium on Hair Follicles Orlando, Florida
July 8-13, 2001	Gordon Conference Epithelial Differentiation Session Chair : Skin Disorders in Mice and Men
September 18, 2001	Symposium on Gene Therapy Uppsala, Sweden
September 20, 2001	European Society of Dermatological Research Postgraduate Course "Molecular Genetics of Hair Disease"
October 20, 2001	World Congress of Pediatric Dermatology "Prenatal Diagnosis of Skin Disorders" Cancun, Mexico
November 17, 2001	Heinrich Heine Universität-Dusseldörf Hautklinik Blistering Diseases: What is new? Dusseldörf, Germany
December 8, 2001	Mt. Sinai Winter Symposium Advances in Medical and Surgical Dermatology "Of hairless mice and men" New York, NY
February 19-24, 2002	Keystone Symposium Genotype to Phenotype: Focus on Disease Invited Speaker "Hairlessness in Mice and Humans" Santa Fe, NM
April 26-27, 2002	Irish Association of Dermatologists Spring Meeting Waterford, Ireland
July 1-5, 2002	20th World Congress of Dermatology Symposium: Genetics and the Skin Symposium: Keratinizing Disorders Paris, France

September 26, 2002	European Society of Veterinary Dermatology Nice, France
February 26, 2003	Department of Dermatology Grand Rounds Columbia University
July 13, 2003	Gordon Conference on Epithelial Differentiation Tilton, NH
October 16, 2003	International Society of Hair Restoration Surgery New York, NY
December 2, 2003	Collaborative Course on Biology of the Skin Boston University Boston, MA
March 1, 2004	Cutaneous Biology Research Center Harvard Medical School Charlestown, MA
June 17, 2004	Hair 2004 Berlin Germany

SOCIETY MEMBERSHIPS

1. Dermatology Foundation 1991 - present.
2. Society for Investigative Dermatology 1991 - present.
3. American Federation for Clinical Research 1994 - present.
4. The New York Academy of Sciences 1994 - present.
5. American Society for Human Genetics 1996 - present.

SCIENTIFIC ADVISORY BOARDS AND TRUSTEE POSITIONS

1. National Association for Pseudoxanthoma Elasticum 1989 - present.
2. D.E.B.R.A. of America 1991 - present.
3. D.E.B.R.A. Scientific Advisory Board 1992 - present.
4. D.E.B.R.A. Board of Trustees 1995 - present.
5. National Alopecia Areata Foundation, Scientific Advisory Board 2003-present.

INTERNAL COMMITTEE APPOINTMENTS

1. Columbia University, Department of Dermatology, Executive Committee - September 1995 - present.

2. Columbia University - Faculty Council of the Faculty of Medicine - July 1, 1997 - June 30, 2000.

3. Columbia University, Curriculum Committee, Subcommittee on Genetics and Genomics, January 1999 - present.

4. Columbia University, Task Force on Human Genetics, January 2001-present.

5. Columbia University, Strategic Planning Steering Committee on Education, September 2001-present.

EXTERNAL COMMITTEE APPOINTMENTS

1. Society for Investigative Dermatology-Albert Kilgman Travel Fellowship Committee - 1996-1999.

2. EPA/NIEHS Liaison Committee Consultant 1997-1998.

3. Joint Committee for the Advancement of the Dermatologic Health of Women - Consultant - 1997- present.

4. Society for Investigative Dermatology-International Investigative Dermatology Travel Fellowship Committee - 1997-1998.

5. Society for Investigative Dermatology-International Investigative Dermatology Ad-Hoc Review/Program Committee - 1997-1998.

6. Dermatology Foundation Medical and Scientific Committee – 2001-2004.

7. Society for Investigative Dermatology, Committee on Scientific Programs, 2001-2005.

8. North American Hair Research Society – Membership Committee – 2001-present.

TRAINEES

At Jefferson Medical College (1991-1995)

1. Xin Zhang, M.S.	Research Associate 1991-1995
2. Yoshiko Tamai, M.D.	Research Associate 1992-1993
3. Yili Xu, M.D.	Research Associate 1993-1995
4. Sabatino Ciatti, M.D.	Resident Fellow 1993-1995
5. Leena Pulkkinen, Ph.D.	Predoctoral Fellow 1992-1994
6. Sal LaForgia, M.D., Ph.D.	Resident Fellow 1994-1995
7. Sirpa Kivirikko, M.D.	Predoctoral Fellow 1992-1995
8. John McGrath, M.D., Ph.D.	Postdoctoral Fellow 1994-1995

At Columbia University (1995-present)

9. Ha Mut Lam	Research Associate 1995-present
10. Peter Cserhalmi, M.D.	Postdoctoral Fellow 1996-present
11. Jorge Frank, M.D.	Postdoctoral Fellow 1996-1999
12. Xiuhua Wang, Ph.D.	Postdoctoral Fellow 1996-1998
13. Kier DeLeo	Predoctoral Fellow May 1996- August 1996
14. Wasim Ahmad, Ph.D.	Postdoctoral Fellow 1997-1999
15. Akua Gyabaah	Predoctoral Fellow June 1997-July1997

16. Kelechi Iheagwara — Predoctoral Fellow June 1997-July1997
17. Julie Grossman — Predoctoral Fellow June 1997-August 1997
18. Douglas Melman, M.D. — Medical Student September-October 1997
19. Jon Nelson, M.D. — Medical Student September-October 1997
20. Andrei Panteleyev, Ph.D. — Postdoctoral Fellow February 1998-present
21. Hendrik Uyttendaele, M.D., Ph.D. — Postdoctoral Fellow 1998-1999
22. Enioma Nwanka — Predoctoral Fellow August 1998
23. Vincent M. Aita, Ph.D. — Postdoctoral Fellow December 1999-Nov 2000
24. Karima Djabali, Ph.D. — Postdoctoral Fellow July 1999-present
25. Marija Tadin — Doctoral Candidate September 1999-present
26. Amalia Marinez Mir, Ph.D. — Postdoctoral Fellow February 2000-present
27. Andrew Engelhard, Ph.D. — Postdoctoral Fellow June 2000-present
28. Ana Kljuic — Doctoral Candidate November 2000-present
29. Ryan O'Shaugnessy, Ph.D. — Postdoctoral Fellow October 2000-present.
30. Hisham Bazzi — Doctoral Candidate September 2002-present
31. Hyunmi Kim — Doctoral Candidate September 2002-present

Graduate Students

Candidate: Marija Tadin
Faculty: Department of Genetics & Development, Columbia University
Thesis Project: Genetics of Hypertrichosis
Thesis Awarded May 14, 2003

Candidate: Ana Kljuic
Faculty: Department of Genetics & Development, Columbia University
Thesis Project: Genetic Studies in the Lanceolate Hair Phenotype
Thesis Awarded (with Distinction) April 4, 2003
Samuel W. Rover and Lewis Rover Award for
Scholarship and Proficiency in Genetics and Development

Candidate: Hisham Bazzi
Faculty: Department of Genetics & Development, Columbia University

Candidate: Hyunmi Kim
Faculty: Department of Genetics & Development, Columbia University

Trainees obtaining independent NIH funding

1. Peter B. Cserhalmi-Friedman, M.D., Ph.D.
NIH K01 Grant "Gene Therapy for DDEB"
NIH K01 AR02183

2. Peter B. Cserhalmi-Friedman, M.D., Ph.D.
NIH R03 Grant "Gene Therapy Strategies for DDEB"
NIH R03 AR47184

3. Andrei A. Panteleyev, Ph.D.
NIH K01 Grant "Functional Analysis of the Hairless Protein"
NIH K01 AR02204

4. Andrei A. Panteleyev, Ph.D.
NIH R03 Grant "Functional Analysis of the Hairless Protein"

NIH R03 AR47403

5. Karima Djabali, Ph.D.
NIH R03 Grant (active)

6. Karima Djabali, Ph.D.
NIH K01 Grant (active)

7. Andrew Engelhard, Ph.D.
NIH R03 Grant (active)

8. Andrew Engelhard, Ph.D.
NIH K01 Grant (pending)

EDUCATIONAL RESPONSIBILITIES

Course Director: DR90P Dermatology Research Elective
Presbyterian Hospital/ Roosevelt Hospital
New York, N.Y.
September 1995 - present.

Course Director: Dermatology Resident Training Program
Journal Club in Genetics
September 1995 -1997.

Participating Lecturer: Dermatology Resident Training Program
Basic Science Lecture Series
"Principles of Genetics" and
"Biology of Collagen and Elastic Tissue"
September 1995 - present.

Guest Lecturer: Brigham and Women's Hospital
Department of Medicine
Collaborative Course in Dermatology
Boston, MA
October 19-20, 1995

Participating Lecturer: G4027Y Principles of Developmental Biology
Spring 1996
Graduate Program in Genetics and Development
Columbia University
"Properties of Epidermal Stem Cells"

Participating Lecturer: M8290 Incorporating Genetics into Adv. Nursing Practice
Fall 1997, 1998
School of Nursing
Columbia University
"Genetic Basis of Disease"

Guest Lecturer: Biochemistry and Molecular Biology
Harvard Medical School
Boston, MA

"Basement Membrane and Diseases of the Skin"
November 18, 1997

Guest Lecturer: Sarah Lawrence College
Bronxville, NY
Genetic Counseling Training Program
November 24, 1997

Participating Lecturer: G4027Y Principles of Developmental Biology
Spring 1998
Graduate Program in Genetics and Development
Columbia University
"Properties of Epidermal Stem Cells"

Guest Lecturer: Memorial Sloan Kettering Medical College
New York, NY
Graduate Student Training Program
September 30, 1999

Guest Lecturer: Brigham and Women's Hospital
Department of Medicine
Collaborative Course in Dermatology
Boston, MA
November 4-5, 1999

Guest Lecturer: Sarah Lawrence College
Bronxville, NY
Genetic Counseling Training Program
February 18, 2000

Participating Lecturer: G4027Y Principles of Developmental Biology
April 13, 2000
Graduate Program in Genetics and Development
Columbia University
"Properties of Epidermal Stem Cells"

Participating Lecturer: G6211Y Genetic Approaches to Biological Problems
April 18, 2000
Graduate Program in Genetics and Development
Columbia University
"Genetics of Human Skin Disease"

Participating Lecturer: Columbia University
College of Physicians & Surgeons
May 5, 8, 2000
Science Basic to the Practice of Medicine and Dentistry
"The Genetic Make-up of an Individual"
"Patterns of Genetic Inheritance"

Guest Lecturer: Memorial Sloan Kettering Medical College
New York, NY
Graduate Student Training Program
September 14, 2000

Participating Lecturer: Columbia University
College of Physicians & Surgeons
September 19-20, 2000 & April 30, 2001
Science Basic to the Practice of Medicine and Dentistry
"The Genetic Make-up of an Individual"
"Patterns of Genetic Inheritance"
"Genomics"
" Structure and Function of the Skin"

Guest Lecturer: Columbia University
Department of Biology
Undergraduate Seminar Series
November 20, 2000

Participating Lecturer: Columbia University
College of Physicians & Surgeons
Second Year Medical Student Course in Dermatology
"Molecular Basis of Skin Disease"
May 23, 2001

Guest Lecturer: Memorial Sloan Kettering Medical College
New York, NY
Graduate Student Training Program
September 13, 2001

THESIS COMMITTEES

Candidate: Poulabi Banerjee (T. Conrad Gilliam Lab)
Faculty: Department of Genetics & Development, Columbia University
Thesis: "Genetic Mapping and Positional Cloning of an Autosomal Recessive Retinitis Pigmentosa (RP14 on 6p21.3) Gene, Tubby-like Protein 1 (TULP1) in Two Extended Kindreds from the Dominican Republic"
Degree Conferred: Doctor of Philosophy, October 9, 1998

Candidate: Anjali Shah (T. Conrad Gilliam Lab)
Faculty: Department of Genetics & Development, Columbia University
Thesis: "Functional Analysis of the Wilson's Disease Gene and Its Role in Copper Transport Disorders"
Degree Conferred: Doctor of Philosophy, March 26, 1999

Candidate:Vincent M. Aita (T. Conrad Gilliam Lab)
Faculty: Department of Genetics & Development, Columbia University
Thesis: "The Mapping of Complex Genetic Traits in Humans"
Degree Conferred: Doctor of Philosophy, November 17, 1999

Candidate: Todd A. Carter (T. Conrad Gilliam Lab)
Faculty: Department of Genetics & Development, Columbia University
Thesis: Positional Cloning of the SMT Gene in Spinal Muscular Atrophy
Degree Conferred: Doctor of Philosophy, January 7, 2000

Candidate: Katerina A. Politi (Argiris Efstradiatis Lab)
Faculty: Department of Genetics & Development, Columbia University
Thesis: Molecular Pathways in Mouse Tumorigenesis

Degree Conferred: Doctor of Philosophy, October 29, 2002

GRANT REVIEWER

Veterans Administration External Reviewer
American Federation for Clinical Research
NIH-National Institute of Arthritis, Musculoskeletal and Skin Diseases (ad hoc)
Dermatology Foundation Medical and Scientific Committee
NIH –Mammalian Genetics Study Section Ad Hoc Reviewer
NIH – General Medicine A-1 (GMA-1) Study Section Ad Hoc Reviewer

MANUSCRIPT REFEREE

American Journal of Human Genetics
American Journal of Pathology
Archives of Dermatology
British Journal of Dermatology
Cancer Research
Clinical Genetics
Development
Developmental Dynamics
Differentiation
European Journal of Human Genetics
Experimental Cell Research
Experimental Dermatology
FASEB Journal
Genomics
Human Genetics
Human Molecular Genetics
Journal of Cell Science
Journal of Clinical Investigation
Journal of the European Academy of Dermatology and Venereology
Journal of Investigative Dermatology
Journal of Investigative Medicine
Laboratory Investigation
Matrix Biology
Mechanisms of Development
Nature
Nature Genetics
Nature Reviews in Cell Biology
Proceedings of the Association of American Physicians
Proceedings of the National Academy of Sciences

PUBLICATIONS

1. Boyd, C.D., Weliky, K., Toth-Fejel, S-E., Deak, S.B., Christiano, A.M., Mackenzie, J.W. Sandell, L.J., Tryggvason, K. and Magenis, E. (1986) The Single Copy Gene Coding for Human Alpha I(IV) Procollagen is Located at the Terminal End of the Long Arm of Chromosome 13. **Hum. Genet.** 74:121-125.

2. Bowcock, A.M., Hebert, J.M., Christiano, A.M., Wijsman, E., Cavalli-Sforza, L.L. and Boyd, C.D. (1987) The Pro Alpha I(IV) Collagen Gene is Linked to the D13S3

Locus at the Distal End of Human Chromosome 13q. **Cytogenet. Cell. Genet.** 45:234-236.

3. Boyd, C.D., Christiano, A.M., Pierce, R.A., Stolle, C.A. and Deak, S.B. (1991) Mammalian Tropoelastin: Multiple Domains of the Protein Define an Evolutionarily Divergent Amino Acid Sequence. **Matrix/ Coll.Rel. Res.** 11:235-241.

4. Sawamura, D., Li, K., Nomura, K., Sugita, Y., Christiano, A.M. and Uitto, J. (1991) Bullous Pemphigoid Antigen: cDNA Cloning, Cellular Expression and Evidence for Polymorphism of the Human Gene. **J. Invest. Derm.** 96:908-915.

5. Tromp, G., Christiano, A.M., Goldstein, N., Indik, Z., Rosenbloom, J., Deak, S.B., Boyd, C.D., Prockop, D.J. and Kuivaniemi, H. (1991) A to G Polymorphism in Exon 20 of the Elastin Gene. **Nucl. Acids Res.** 19:4314.

6. Ryynänen, J., Sollberg, S., Parente, M.G., Chung, L.C., Christiano, A.M. and Uitto, J. (1992) Type VII Collagen Gene Expression by Cultured Human Cells and in Fetal Skin. **J. Clin. Invest.** 89:163-168.

7. Christiano, A.M., Rosenbaum, L.M., Chung-Honet, L.C., Parente, M.G., Woodley, D.T., Pan, T.C., Zhang, R.Z., Chu, M.L., Burgeson, R.E. and Uitto, J. (1992) The Large Non-Collagenous Domain (NC-1) of Human Type VII Collagen is Amino-Terminal and Chimeric: Homology to Cartilage Matrix Protein, The Type III Domains of Fibronectin, and the A Domain of von Willebrand Factor. **Human Molec. Genet.** 1:475-481.

8. Christiano, A.M., Chung-Honet, L.C., Hovnanian, A. and Uitto, J. (1992) PCR-Based Detection of Two Exonic Polymorphisms in the Human Type VII Collagen Gene (COL7A1) at 3p21.1. **Genomics** 14: 827-828.

9. Pulkkinen, L., Christiano, A.M., Knowlton, R.G. and Uitto, J. (1993) Epidermolytic Hyperkeratosis (Bullous Congenital Ichthyosiform Erythroderma): Genetic Linkage to the Type II Keratin Gene Cluster on Chromosome 12q. **J. Clin. Invest.** 91:357-361.

10. Christiano, A.M., Greenspan, D.S., Hoffman, G.G., Zhang, X., Tamai, Y., Lin, A.N., Dietz, H.C., Hovnanian, A. and Uitto, J. (1993) A Missense Mutation in the Human Type VII Collagen Gene in Two Siblings With Recessive Dystrophic Epidermolysis Bullosa. **Nature Genet.** 4:62-66.

11. Li, K., Christiano, A.M., Copeland, N.G., Gilbert, D.J., Chu, M.-L., Jenkins, N.A., and Uitto, J. (1993) cDNA Cloning and Chromosomal Mapping of the Mouse Type VII Collagen Gene (COL7A1): Evidence for Rapid Evolutionary Divergence of the Gene. **Genomics** 16:733-739.

12. Lapiere, J-C., Woodley, D.T., Parente, M.G., Wynn, K.C., Christiano, A.M. and Uitto, J. (1993) Epitope Mapping of Type VII Collagen: Identification of Discrete Peptide Sequences Recognized by Sera From Patients with Acquired Epidermolysis Bullosa. **J. Clin. Invest.** 92: 1831-1839.

13. Hoffman, G.G., Lee, S., Christiano, A.M., Chung-Honet, L.C., Cheng, W., Katchman, S., Uitto, J. and Greenspan, D.S. (1993) Complete Coding Sequence, Intron/Exon Organization and Chromosomal Location of the Gene for the Core I

Protein of Human Ubiquinol-Cytochrome C Reductase. **J. Biol. Chem.** 268: 21113-21119.

14. Hilal, L., Rochat, A., Duquesnoy, P., Blanchet-Bardon, C., Wechsler, J., Martin, N., Christiano, A.M., Barrandon, Y., Uitto, J., Goossens, M. and Hovnanian, A. (1993) A Homozygous Frameshift Mutation in COL7A1 Predicting a Shortened Protein in the Generalized Mutilating (Hallopeau-Siemens) form of Recessive Dystrophic Epidermolysis Bullosa. **Nature Genet.** 5:287-293.

15. Christiano, A.M. and Uitto, J. (1994) Heterogeneity of Mutations in the Type VII Collagen Gene in Recessive Dystrophic Epidermolysis Bullosa. **Chron. Derm.** 4:1-12.

16. Lebwohl, M.G., Neldner, K., Pope, F.M., de Paepe, A., Christiano, A.M., Boyd, C.D., Uitto, J. and McKusick, V.A. (1994) Classification of Pseudoxanthoma Elasticum. **J. Am. Acad. Derm.** 30: 103-107.

17. Kalinke, D-U., Kalinke, U., Winberg, J-O., König, A., Lauharanta, J., Christiano, A.M., Uitto, J. and Bruckner-Tuderman, L. (1994) Collagen VII in Severe Recessive Dystrophic Epidermolysis Bullosa: Expression of mRNA but Lack of Intact Protein Product in Skin and Cutaneous Cells in Vitro. **J. Invest. Derm.** 102:260-262.

18. Rudnicka, L., Varga, J., Christiano, A.M., Iozzo, R.V., Jimenez, S.A. and Uitto, J. (1994) Elevated Expression of Type VII Collagen in the Skin of Patients with Systemic Sclerosis: Regulation by Transforming Growth Factor-ß. **J. Clin. Invest.** 93:1709-1715.

19. Pulkkinen, L., Christiano, A.M., Airenne, T., Haakana, H., Tryggvason, K., Uitto, J. (1994) Mutations in the γ2 Chain Gene (LAMC2) of Kalinin/Laminin 5 in the Junctional Forms of Epidermolysis Bullosa. **Nature Genet.** 6:293-298.

20. Aberdam, D., Galliano, M-F., Vailly, J., Pulkkinen, L., Bonifas, J., Christiano, A.M., Tryggvason, K., Uitto, J., Epstein, E., Ortonne, J-P., Meneguzzi, G. (1994) Herlitz's Junctional Epidermolysis Bullosa is Linked to Mutations in the Gene (LAMC2) for the γ2 Subunit of Nicein/Kalinin (Laminin 5). **Nature Genet.** 6:299-304.

21. Chan Y-M., Yu, Q-C., Christiano, A.M., Uitto, J., Kucherlapati, R.S., LeBlanc-Strasecki, J. and Fuchs, E. (1994) Mutations in the Non-Helical Linker Segment L1-2 of Keratin 5 in Patients with Weber-Cockayne Epidermolysis Bullosa. **J. Cell Sci.** 107:765-777.

22. Christiano, A.M., Ryynänen, M. and Uitto, J. (1994) Dominant Dystrophic Epidermolysis Bullosa: Identification of a Glycine-to-Serine Substitution in the Triple-Helical Domain of Type VII Collagen. **Proc. Natl. Acad. Sci. USA** 91:3549-3553.

23. Christiano, A.M., Anhalt, G., Gibbons, S., Bauer, E.A. and Uitto, J. (1994) Premature Termination Codons in the Type VII Collagen Gene (COL7A1) Underlie Severe, Mutilating Recessive Dystrophic Epidermolysis Bullosa. **Genomics** 21:160-168.

24. Christiano, A.M., Hoffman, G., Chung-Honet, L.C., Lee, S., Cheng, W., Uitto, J. and Greenspan, D.S. (1994) Structural Organization of the Human Type VII

Collagen Gene (COL7A1), Comprised of More Exons than Any Previously Characterized Gene. **Genomics** 21:169-179.

25. Hovnanian, A., Hilal, L., Blanchet-Bardon, C., de Prost, Y., Christiano, A.M., Uitto, J. and Goossens, M. (1994) Recurrent Nonsense Mutations within Type VII Collagen in Patients with Severe Mutilating Recessive Dystrophic Epidermolysis Bullosa. **Am. J. Hum. Genet.**55:289-296.

26. Christiano, A.M., Greenspan, D.S., Lee, S. and Uitto, J. (1994) Cloning of Human Type VII Collagen: Complete Primary Sequence of the α1(VII) Chain and Identification of Intragenic Polymorphisms. **J. Biol. Chem.** 269:20256-20262.

27. Baudoin, C., Miquel, C., Gagnoux-Palacios, L., Pulkkinen, L., Christiano, A.M., Uitto, J., Tadini, G., Ortonne, J-P. and Meneguzzi, G. (1994) A Novel Homozygous Nonsense Mutation in the LAMC2 Gene in Patients with the Herlitz Junctional Epidermolysis Bullosa. **Hum. Molec. Genet.** 3:1909-1910.

28. Pulkkinen, L., Christiano, A.M., Gerecke, D., Wagman, D.W., Burgeson, R.E., Pittelkow, M. and Uitto, J. (1994) A Homozygous Nonsense Mutation in the ß3 Chain Gene of Laminin 5 (LAMB3) in Herlitz Junctional Epidermolysis Bullosa. **Genomics** 24:357-360.

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SUBMITTED PUBLICATIONS & MANUSCRIPTS IN PREPARATION

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CHAPTERS IN BOOKS AND REVIEW ARTICLES

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INVENTIONS AND PATENTS

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Human Hairless Gene, Protein and Uses Thereof
Inventor: Christiano, A.M.

U.S.Patent (pending)
Nucleic Acids for Inhibiting Hairless Protein Expression and Methods of Use Thereof
Inventor: Christiano, A.M.

MOLECULAR GENETICS OF EPIDERMOLYSIS BULLOSA 10/21/03

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INTRODUCTION

Significant progress has recently been made in understanding the structural features of the epidermis and the dermal–epidermal junction, largely through molecular cloning of genes that encode proteins critical for the structural integrity of these cutaneous layers. Disturbances in the expression of the genes within the stratified layers of the dermal–epidermal adhesion zone provide the basis for different forms of heritable blistering skin diseases with divergent clinical presentations. This overview highlights epidermolysis bullosa (EB) as the paradigm of heritable disorders of the basement membrane zone and illustrates the power of molecular diagnostics in blistering skin diseases by reviewing recent revelations into the molecular basis of different forms of EB.

MOLECULAR COMPLEXITY OF THE CUTANEOUS BASEMENT MEMBRANE ZONE

Electron microscopic examination of the cutaneous basement membrane zone (BMZ) reveals the presence of several attachment complexes, critical for integrity of the stable association of epidermis and dermis (Fig. XX-1). These include hemidesmosomes that extend from the intracellular compartment of the basal keratinocytes to the lamina lucida in the upper portion of the dermal–epidermal basement membrane. Within the lamina lucida, the hemidesmosomes interact with anchoring filaments, thread-like structures that tend to concentrate below the

hemidesmosomes. At the lower portion of the dermal–epidermal attachment zone, fibrillar structures known as anchoring fibrils extend from the lamina densa of the basement membrane to the papillary dermis, where they may associate with basement-membrane-like structures known as anchoring plaques. Alternatively, they may re-insert into the lamina densa forming U-shaped structures that entrap interstitial collagen fibers in the upper papillary dermis and secure the association of lamina densa with the upper dermis.

Molecular cloning and biochemical analyses of the cutaneous BMZ components have allowed elucidation of the structural and functional characteristics of numerous macromolecules which constitute these dermal–epidermal attachment complexes. First, hemidesmosomes have been shown to consist of at least four distinct proteins: (a) the 230-kDa bullous pemphigoid antigen (BPAG1), a noncollagenous protein that serves as an autoantigen in the acquired autoimmune disease bullous pemphigoid; (b) the 180-kDa bullous pemphigoid antigen (BPAG2), a transmembrane collagenous protein, also known as type XVII collagen (COL17A1); (c) basal keratinocyte-specific integrin $\alpha6\beta4$, which contributes to the anchoring of basal keratinocytes to the underlying basement membrane; and (d) plectin, a ~500-kDa adhesion molecule belonging to the plakin family of proteins. Secondly, the anchoring filaments, which traverse the lamina lucida, have been shown to consist of laminin 5, a distinct member of the laminin family of proteins. Laminin 5 consists of three constitutive subunit polypeptides, the $\alpha3$, $\beta3$, and $\gamma2$ chains, which form a characteristic cross-shaped trimeric structure. Finally, type VII collagen is the major, if not the exclusive, component of anchoring fibrils. The primary structure of type VII collagen has been determined through cDNA cloning, and the intron–exon organization of the entire gene has been elucidated.

Collectively, the data summarized above indicate that the cutaneous BMZ is a complex

continuum of interacting macromolecules that form a network securing the stable association of the epidermis with the underlying dermis. Thus, genetic mutations resulting in abnormalities in any component of this network could result in a blistering skin disease, such as the variants of EB.

MOLECULAR BASIS OF DIFFERENT FORMS OF EPIDERMOLYSIS BULLOSA

The prototype of the diseases affecting the cutaneous BMZ is EB, a group of heritable mechanobullous disorders that manifest with considerable variability in clinical presentation and severity (Fig. XX-2). EB is also genetically heterogeneous, as both autosomal dominant and autosomal recessive forms of EB can be recognized. Traditionally, EB has been divided into three broad categories based on the level of tissue separation within the cutaneous BMZ, as established by diagnostic transmission electron microscopy or by immunoepitope mapping (Table XX-I). In the simplex forms of EB (EBS), the tissue separation occurs within the basal keratinocytes, primarily as a result of mutations in the basal keratin genes, *KRT5* and *KRT14*. In the junctional forms of EB (JEB), the tissue cleavage occurs within the dermal–epidermal basement membrane at the level of the lamina lucida, and the hemidesmosome-anchoring filament complexes demonstrate ultrastructurally detectable abnormalities. In patients with the classic forms of JEB, specific mutations have been identified in the genes, *LAMA3*, *LAMB3* and *LAMC2*, which encode the constitutive subunit polypeptides, α3, β3, and γ2, of the anchoring-filament protein laminin 5, respectively. In the dystrophic forms of EB (DEB), the tissue separation occurs below the lamina densa at the level of anchoring fibrils, and the mutations reside in the type VII collagen gene, *COL7A1*. In addition to the traditional classification – simplex, junctional, and dystrophic – we have recently introduced the fourth category, the

hemidesmosomal variants, the molecular pathology involving the hemidesmosomal proteins (Table XX-I). These include three clinically distinct variants of EB, (a) generalized atrophic benign EB (GABEB), (b) EB with pyloric atresia (EB-PA), and (c) EB with muscular dystrophy (EB-MD). The corresponding mutations were identified in the genes encoding the 180-kDa bullous pemphigoid antigen, *BPAG2*, also known as type XVII collagen; the subunit polypeptides of the $\alpha 6\beta 4$ integrin; and plectin, a large adhesion molecule expressed in hemidesmosomes as well as in the sarcolemma of the muscle.

In addition to their role in inherited EB, several of these proteins serve as autoantigens in acquired bullous skin diseases of adulthood, such as bullous pemphigoid, herpes gestationis and paraneoplastic pemphigus, and unusual mutations in a few of them even result in inherited diseases that fall outside the spectrum of EB.

THE SIMPLEX FORMS OF EB – MUTATIONS IN THE BASAL KERATINS, KRT5 AND KRT14

Keratin proteins, the major constituents of epithelial cells, represent a large family of about 50 proteins that form 10 nm keratin intermediate filaments (KIF) and are expressed in tissue- and differentiation-specific patterns. KIF built a dense, three-dimensional and highly dynamic cytoskeleton spanning between the nucleus and the cell membrane, which provides structural stability and flexibility and ensures the mechanical integrity of the epithelial tissues (Fig. XX-3, Top). Keratins are expressed in pairs of acidic (type I) and basic (type II) proteins, the genes for which cluster on chromosomes 17q12-q21 and 12q11-q13. Keratin monomers are organized as a central, alpha-helical rod of about 310 amino acids, flanked by variable, non-helical head and tail domains (Fig. XX-3, Bottom). The monomers form coiled-coil obligate heterodimers, which

polymerize in overlapping and antiparallel fashion and assemble into intermediate filaments. In the epidermis, undifferentiated basal keratinocytes express the principal keratins K5 (type II) and K14 (type I), and to a lesser extent K15 (type I), while cells in the upper epidermis switch to the expression of the differentiation-specific keratin pair K1/K10, and in the granular layers also K2e. Other site-specific suprabasal keratins include K9 found predominantly at palms and soles, and K6, K16 and K17, which are induced by trauma to the skin. Pathogenic mutations in 19 different keratin genes have been discovered so far in a wide range of epithelial fragility disorders affecting skin, mucous membranes, hair, nails, and sebaceous glands, whereby the disease pathology, in general, corresponds to the expression pattern of the defective keratin proteins.

EBS was the first human keratin disorder to be discovered in 1991 and has become the prototype for understanding pathogenesis and genotype-phenotype correlations within this broad group of disorders. EBS is a clinically heterogeneous, congenital blistering disorder due to fragility of basal cells of the epidermis. Three major subtypes have been recognized and share a common disease pathology with an abnormal KIF network, cytolysis of basal cells leading to intraepidermal blister formation and skin fragility, while the differentiation of keratinocytes in the upper layers of the epidermis remains largely undisturbed. The structural abnormalities confined to the basal compartment of the epidermis and mucous membranes result from faulty K5/K14 heterodimers due to mutations in their corresponding genes, *KRT5* and *KRT14*. Mutations in either gene can produce a comparable phenotype reflecting the genetic heterogeneity of EBS. *KRT5* and *KRT14* mutations are in general autosomal dominant and lead to replacement of conserved amino acid residues, which has a deleterious, dominant-negative effect on the assembly of KIF and causes weakening of the cytoskeleton and cell fragility.

To date, over 80 distinct pathogenic mutations have been identified in EBS. They are clustered at 7 defined regions (Fig. XX-3, Bottom), and their positions seem to dictate the severity of disease. The majority of mutations (~60%) occur at two "hot spots" corresponding to the beginning and end of the central rod domain, known as helix initiation peptide and helix termination peptide. Functional *in vivo* and *in vitro* studies have demonstrated that the helix boundaries are zones of overlap between keratin heterodimers during filament assembly and that mutant keratin molecules perturb proper keratin alignment, and thereby oligomerization, filament assembly and integrity (Fig. XX-3, Top). Consequently, these "hot spot" mutations are consistently associated with a severe phenotype. Other mutation sites, such as the homologous domain in the head of type II keratins or non-helical linker and stutter motifs, are associated with a milder phenotype. The former region has been implicated in filament assembly and contains major phosphorylation sites while the latter regions provide crucial flexibility to the otherwise rigid alpha-helical rod. In addition, a few autosomal recessive mutations leading to the 'knock-out' of a keratin in the basal epidermis have also been described in EBS.

EBS Dowling-Meara (EBS-DM; OMIM 131760) is a common and the most severe subtype manifesting at birth with erythema, widespread blistering, erosions and areas of denuded skin (Fig. XX-2). While spontaneous development of grouped blisters at any body site slowly improves with age, a progressive palmoplantar keratoderma becomes the chief complaint in adulthood. Other features of EBS-DM include involvement of mucous membranes, secondary skin infections and sepsis, nail dystrophy, milia, and healing with hypo- and hyperpigmentation of the skin. Typically, pathogenic defects are heterozygous missense mutations clustering at the highly conserved boundaries of the alpha-helical rod of K5 or K14. An arginine codon of the helix initiation peptide in K14 (R125) is most commonly mutated, in >30% of patients, probably

because it contains a hypermutable CpG dinucleotide. As a result, the arginine codon (CGC) is replaced either by one for cysteine (TGC) or histidine (CAC). While most keratin gene mutations in EBS are dominant, recessive *KRT14* mutations have been identified in seven families, encompassing predominantly nonsense and splice site mutations leading to premature termination of protein translation and ablation of the affected keratin. While the 'knock-out' of *KRT14* usually results in severe EBS-DM, mutations in three families were noted to have a milder phenotype with minimal extracutaneous involvement.

The Köbner subtype of EBS (EBS-K; OMIM 131900) is characterized by milder, generalized blistering of the skin without apparent clustering, often in response to minor trauma and induced by increased ambient temperature. Hands, feet and extremities are most consistently affected, probably because of the greater mechanical trauma at these body sites. *KRT5* or *KRT14* mutations appear more widely distributed across the keratin polypeptides and may lie within and outside the highly conserved helix boundaries as well as in non-helical linker segments.

EBS Weber-Cockayne (EBS-WC; OMIM 131800) is the most common, relatively mild, localized subtype of EBS, in which serous blisters are confined to the hands, feet, and areas of friction or trauma. In contrast to the other EBS subtypes, blisters are usually not present at birth but develop later in life in response to mechanical trauma to the skin. The disorder worsens with sweating and at increased ambient temperature. Ultrastructural abnormalities of the cytoskeleton are far less severe than those seen in EBS-DM, and even essentially normal KIF ultrastructure has been reported. In this relatively mild form of EBS, pathogenic mutations lie in most cases outside of the helix boundaries, elsewhere in the rod domain of K5 or K14, including the non-helical L12 linker motif or in the amino-terminal homologous domain of K5. Dominant

point mutations usually result in amino acid substitutions, but a small in-frame deletion in *KRT14* has also been described.

EBS with mottled pigmentation (EBS-MP; OMIM 131960) is a rare form of EBS with generalized or acral blistering and small hyper- and hypopigmented spots that form a reticulate pattern. This variant is also associated with thickened, dystrophic nails and punctate palmoplantar keratoderma. The mottled pigmentation seems unrelated to skin blistering and corresponds with an increased number of melanosomes within basal keratinocytes, dermal macrophages and Schwann cells, as observed by electron microscopy. EBS-MP is caused by a heterozygous missense mutation in exon 1 of *KRT5* that results in substitution of proline 24 with leucine (P24L) in the non-helical head domain of K5. This mutation was consistently detected in each of seven unrelated families with EBS-MP tested to date. Preliminary investigations of this mutation revealed that the keratin 5 head domain may directly bind to a cytoplasmic dynein cargo complex transporting melanosomes, thus unraveling the basis for abnormal pigment distribution in this rare disorder.

MOLECULAR BASIS OF THE JUNCTIONAL FORMS OF EB (JEB)

The junctional forms of EB display a remarkable degree of genetic heterogeneity, with seven different genes implicated in its pathogenesis thus far. In JEB, tissue cleavage occurs within the dermal-epidermal basement membrane at the level of the lamina lucida or the overlying hemidesmosomes, and ultrastructural abnormalities are evident in the hemidesmosome (HD)-anchoring filament (AF) complexes.

The Hemidesmosome-Anchoring Filament Complex

The hemidesmosomes extend from the intracellular compartment of the basal keratinocytes to the lamina lucida in the upper portion of the dermal–epidermal basement membrane. Within the lamina lucida, the hemidesmosomes attach to anchoring filaments, thread-like structures that tend to concentrate below the hemidesmosomes. Early biochemical studies identified five major components of the HDs, consisting of polypeptides with molecular masses of 500, 230, 200, 180, and 120 kDa; these were originally designated as HD1–HD5, respectively. HD2 and HD4 have since been shown to be identical to the 230-kDa bullous pemphigoid antigen (BPAG1) and the180-kDa bullous pemphigoid antigen (BPAG2/COL17A1), respectively. HD3 and HD5 correspond to the β4 and α6 integrins, respectively, and HD1 corresponds to plectin. Plectin is localized to the cytoplasmic side of the HDs, in a distribution slightly above, yet almost indistinguishable from BPAG1, at the level of the cytoplasmic periphery of the HD inner plaque. The intracellular hemidesmosomal plaque is comprised of the 230-kDa bullous pemphigoid antigen (BPAG1), a non-collagenous protein of the plakin family, that serves as an autoantigen in an acquired autoimmune disease, bullous pemphigoid. The 180-kDa bullous pemphigoid antigen (BPAG2), a transmembrane collagen also known as type XVII collagen (COL17A1), together with α6β4 integrin, extends from the intracellular compartment into the extracellular space, thus stabilizing the association of basal keratinocytes to the underlying basement membrane. Consequently, mutations resulting in abnormalities in any one of the protein components of the HD-AF network can give rise to the different forms of JEB.

Molecular Heterogeneity of Junctional EB

The junctional forms of EB display a considerable range of phenotypic heterogeneity, and on

the basis of clinical severity, the disease has been traditionally divided into the classic, Herlitz (lethal) type (OMIM 226700), and a variety of non-Herlitz (non-lethal) forms (OMIM 226650). Within the non-lethal forms, several subtypes have been described based on the associated extracutaneous manifestations and the extent and severity of the blistering tendency. Three of these subtypes traditionally classified as non-Herlitz JEB have now been considered as the hemidesmosomal variants of EB (see below). This clinical heterogeneity of JEB reflects the repertoire of underlying genetic lesions demonstrated thus far in as many as seven different genes. Mutations in different forms of JEB (including the hemidesmosomal variants) have now been identified in the each of the known genes encoding the macromolecular components of the HD-AF complex, with the exception of BPAG1. Careful examination of the mutation database in these genes reveals genotype-phenotype correlations which reflect the expression of the mutated genes, the types and combinations of the mutations, and the consequences at the mRNA and protein levels.

Molecular Basis of Herlitz JEB: Premature Termination Codon Mutations in the Laminin 5 Genes

Historically, electron microscopic studies on the skin of JEB patients first revealed ultrastructural abnormalities in the HD-AF complexes, and specifically, the hemidesmosomes were found to be rudimentary and poorly formed. In the most severe, clinically devastating Herlitz type of JEB, immunofluorescence staining for laminin 5 epitopes suggested the complete absence of this protein. Subsequent to the cloning of genes encoding the three constitutive polypeptides of laminin 5, mutation detection strategies have since revealed genetic mutations in each of the three genes, *LAMA3*, *LAMB3*, and *LAMC2*, encoding the α3, β3, and γ2 chains,

respectively. The majority of these mutations are located in the LAMB3 gene, due to the presence of two mutational hotspots (R635X and R42X) leading to recurrent mutations. It is noteworthy that in the Herlitz form of JEB, all mutations disclosed thus far result in premature termination codons in one of the laminin 5 chains, leading to markedly reduced levels of the corresponding mRNA transcript via nonsense-mediated mRNA decay, and the virtual absence of the corresponding protein.

Molecular Basis of Non-Herlitz JEB: Compound Heterozygous Mutations in the Laminin 5 Genes

Laminin 5 gene mutations have been also discovered in the some of the non-lethal forms of JEB (OMIM 226650), however the type and combinations of mutations specify a milder clinical phenotype. In some cases, only one of the mutations is a premature termination codon in one of the laminin 5 genes. However, the other genetic lesion may consist of a missense mutation or an in-frame exon-skipping mutation, each of which would encode for some laminin 5 protein, albeit with impaired function. These observations suggest that polypeptides with an intact carboxy-terminal end are able to assemble into trimer molecules, which serve a partial function in the anchoring filaments. This interpretation is consistent with the observation that immunofluorescence microscopy performed with an anti-laminin 5 antibody, such as GB3, is positive, although frequently attenuated, in the skin of non-lethal JEB patients.

It should be noted that *LAMA3* mutations have recently been disclosed in laryngo-onycho-cutaneous (LOC) syndrome or Shabbir syndrome (OMIM 245660), a recessively inherited disorder with some features of skin fragility. The disorder is typified by skin erosions, nail dystrophy and exuberant granulation tissue in specific epithelia, particularly the conjunctiva and

larynx. Linkage mapping localized the gene to chromosome 18q11.2 in a region including the laminin α3 gene (*LAMA3*). By further analysis, it was noted the LAMA3 gene encodes three distinct proteins generated by alternative promoter utilization and internal splicing, which are designated laminin α3a, α3b1 and α3b2. The identical causative mutation in 15 LOC families was found to be a frameshift mutation that is specific only to the laminin α3a isoform. Thus, in LOC, only one LAMA3 isoform is affected, while in Herlitz JEB, the mutations reside in a commonly utilized region among the three isoforms, therefore leading to complete absence of all LAMA3 chains. These studies provide insight into the specific role of the N-terminal domain of laminin α3a in the granulation tissue response noted in Herlitz-JEB.

MOLECULAR BASIS OF THE HEMIDESMOSOMAL VARIANTS OF JEB

Whereas the molecular basis of many of the classic forms of Herlitz and non-Herlitz JEB are now well-defined on the basis of laminin 5 mutations, there is a group of patients in whom blisters form in and around the hemidesmosome (HD), and have therefore been referred to as the "hemidesmosomal variants". Ultrastructural classification initially identified at least three groups of patients with EB whose blisters form at the level of the HD, and whose clinical phenotype is unlike any of the 'classic' forms of EB. These three subtypes include generalized atrophic benign epidermolysis bullosa (GABEB, OMIM 226650), epidermolysis bullosa with pyloric atresia (EB-PA, OMIM 226730), and epidermolysis bullosa with muscular dystrophy (EB-MD, OMIM 226670). Mutations have recently been identified in three HD-associated structural proteins/genes in patients with these rare forms of EB.

Mutations in BPAG2 in Generalized Atrophic Benign EB

Underscoring the genetic heterogeneity of the junctional forms of EB is the demonstration of specific mutations in genes encoding structural components of the hemidesmosomes. Specifically, mutations have been identified in the *COL17A1* gene in a subset of patients originally classified as a non-lethal JEB variant, known as generalized atrophic benign EB (GABEB). Clinically, these patients demonstrate a moderate blistering tendency, associated with a characteristic constellation of extracutaneous involvement, including dystrophy of the fingernails, focal scarring alopecia of the scalp, loss of eyelashes, dental anomalies, and patchy macular hyperpigmentation. The mutation in the original GABEB family, described in a geographically limited area in Austria, is a premature termination codon transmitted through many branches of the large inbred family. Subsequently, many different types of mutations in *COL17A1* have been observed in GABEB, including missense, nonsense, splice-site and insertions/deletions, resulting in premature termination codons. While there is slight variation in phenotypic severity, GABEB is uniformly non-lethal, and there does not appear to be a strict genotype-phenotype correlation with respect to *COL17A1* mutations, as compared with the laminin 5 genes. Recently, a dominantly inherited glycine substitution mutation in *COL17A1* was identified in a GABEB family, in which the proband had inherited a premature termination codon on the second allele. Interestingly, all heterozygous carriers of the glycine substitution alone had markedly abnormal dentition and enamel pitting. Clearly, the glycine substitution mutation in COL17A1 also impacts upon the basement membrane of the developing tooth, a previously undisclosed function for this transmembrane collagen.

Identification of α6β4 Integrin Mutations in EB with Pyloric Atresia

Another rare subtype of EB, characterized by blistering of the skin and congenital pyloric atresia (EB-PA), was previously classified as a non-lethal form JEB, although the phenotypes can range from moderate to early postnatal demise. Mutations in the genes encoding the two polypeptides of the α6β4 integrin receptors (*ITGA6* and *ITGB4*) have been identified in EB-PA. A survey of the reported mutations indicates that premature termination codons are associated predominantly with the lethal EB-PA variants, whereas missense mutations are more prevalent in non-lethal forms. However, the consequences of the missense mutations are position dependent, and substitutions of highly conserved amino acids within the individual integrin receptors may have lethal consequences. The phenotypic manifestation of pyloric atresia from mutations in the *ITGB4* and *ITGA6* genes suggest a tissue-specific role for this integrin both in the skin and the gastrointestinal tract.

Plectin Mutations in EB with Muscular Dystrophy

To determine which of the candidate proteins of the HD (BPAG1 or plectin) is responsible for making the connection between the HD and the keratinocyte intermediate-filament network, Guo and colleagues (1995) created a knockout mouse in which they targeted *BPAG1*. Whereas the HDs in these mice were largely normal, they lacked the inner plate and demonstrated a complete severance of the connection between the HD with the intermediate-filament network. The zone of mechanical fragility of the basal keratinocytes was restricted to the region of the HD, quite distinct from the ultrastructural findings in classic EB simplex, yet strikingly similar to the

cleavage plane reported in 'pseudo-junctional' EB. Unexpectedly, the *BPAG1* knockout mice also developed late-onset muscular dystonia and neurodegeneration, and was found to be allelic to a naturally occurring mouse, dystonia musculorum (dt). This constellation of phenotypic features together with the ultrastructural findings was highly reminiscent of the pathogenesis of EB with muscular dystrophy. On the basis of the striking similarities in the clinical phenotype of EB with muscular dystrophy and the *BPAG1* knockout mouse, it appeared likely that one of the two components of the hemidesmosomal inner plaque, BPAG1 or HD-1, would be involved in the pathogenesis of this disorder. Subsequently, BPAG1 was ruled out as the cause of EB-MD, and it remains the only known hemidesmosomal component without an associated human genetic disease.

HD-1 was first described as a ~500-kDa component of the hemidesmosome, and was subsequently shown to be identical with plectin, an exceptionally large intermediate filament interacting protein, that was cloned independently. Plectin is a plakin family member with similarities to both desmoplakin and bullous pemphigoid antigen, is highly conserved between rat and human, and has a wide tissue distribution, including the central nervous system and muscle. Plectin has a remarkable number of versatile binding affinities for other proteins, including vimentin, glial fibrillary acidic protein, the neurofilament protein triplet, keratin 5 and 14, and lamin B, suggesting that it can tether one filamentous network to another. Although plectin is expressed in nearly all cell types, its precise cytoplasmic localization is cell-type specific, and it can appear diffuse throughout the cell as a cytomatrix component, or in a restricted distribution as a focal adhesion protein.

Initial examination of biochemical and synthetic properties of cultured keratinocytes from patients with EB-MD using antibodies against plectin showed that this protein was completely

absent in the cells of several patients with EB-MD. Typical presentation of EB-MD is neonatal blistering and late-onset muscular dystrophy with nail and tooth abnormalities. Severe mucocutaneous involvement, including laryngeal webs and urethral strictures, has also been reported. While EB-MD is rare, most of the mutations reported thus far result in premature termination codons on both alleles of plectin, either by nonsense, insertion/deletion or splicing mutations, and the phenotype in these patients is remarkably consistent manifesting with neonatal blistering and progressive muscle weakness from the first or second decade of life on. In some patients, small in-frame deletions or insertions have been disclosed with significantly milder phenotype and the onset of the muscle weakness on the third or fourth decade of life. Thus, continued identification of plectin mutations in patients with EB-MD will provide further insights into the phenotype-genotype correlations in this disorder as well as the relationship between the skin, the musculoskeletal and the nervous systems.

The Ogna Variant of EB is Caused by a Plectin Missense Mutation

While several mutations in the plectin gene have been identified in recessively inherited EB-MD, a different type of mutation in plectin leads to a rare, dominant form of EB, known as the Ogna variant (OMIM 131950). The EB Ogna phenotype is due to a specific missense mutation (R2110W) within the rod domain of plectin. It has since been shown that EB Ogna is not restricted to a single Norwegian kindred as previously believed, since a German family with this phenotype was found to carry the identical mutation. Clinically, the EB Ogna patients demonstrate hemorrhagic blistering of the skin, but unlike patients with EB-MD, there is no muscle phenotype, and muscle biopsies from several EB Ogna patients revealed normal staining patterns with antibodies against plectin. Plectin is one of the largest and most versatile cytolinker

proteins described, which connects the intermediate filament network to the hemidesmosomes in basal keratinocytes. Clearly, the genotype-phenotype relationships among plectin mutations in the different forms of EB are unusually complex and still emerging.

In summary, the junctional forms of EB, including the hemidesmosomal variants, reflect mutations in at least seven different genes disclosed to date, and the specific clinical constellations result from the combination of different types of mutations within the mutated genes (Table XX-1). Whereas the mechanistic consequences of these mutations have been explored at the mRNA and protein levels in many of the studies cited, the functional role of these components in HD assembly is also becoming increasingly clear through recent biochemical studies of protein–protein interactions. For example, an interaction between the 180-kDa bullous pemphigoid antigen and α6 integrin has been described, the disruption of HD assembly by a tailless β4 integrin subunit was reported, and roles for the integrin receptors in mediating signal transduction continue to emerge. Whereas these components clearly represent functionally interdependent structural macromolecules in the dermal–epidermal adhesion zone, recent studies suggest a functionally dynamic and interactive role of some of these proteins in cell-matrix communication. Finally, it is noteworthy that despite an extensive survey of the seven genes involved in JEB, several families have proven negative for mutations, suggesting additional, as yet undisclosed, candidate genes and uncharacterized protein components for this increasingly heterogeneous group of disorders.

THE DYSTROPHIC FORMS OF EB: MUTATIONS IN COL7A1 RESULT IN A SPECTRUM OF CLINICAL SEVERITY

The dystrophic forms of EB (DEB), which can be inherited in either an autosomal dominant or autosomal recessive pattern, demonstrate extensive variability in the clinical spectrum of severity. The less severe forms, such as dominantly inherited dystrophic EB (DDEB) or the mitis type of recessively inherited DEB (M-RDEB), are characterized by a significant blistering tendency, but they lack the extensive mutilating scarring that is the hallmark of the severe, generalized, Hallopeau-Siemens type of recessive dystrophic EB (HS-RDEB). In addition to cutaneous manifestations, the dystrophic forms, particularly HS-RDEB, are associated with scarring of the esophagus and corneal erosions. Furthermore, the patients with HS-RDEB develop unusually aggressive, rapidly metastasizing squamous cell carcinomas primarily in the hand and feet. Thus, the combination of cutaneous and extracutaneous manifestations is associated with considerable morbidity and mortality in the most severely affected patients with DEB.

Molecular Genetics of Dystrophic EB

Several lines of evidence initially suggested that type VII collagen is the candidate gene/protein system harboring mutations in the dystrophic forms of EB. First, the ultrastructural diagnostic hallmark of the dystrophic forms of EB is an abnormality in anchoring fibrils, attachment structures that are composed predominantly, if not exclusively, of type VII collagen. In different variants of DEB, the anchoring fibrils can be shown by transmission electron microscopy to be morphologically abnormal, reduced in number, or even completely absent. Secondly, these ultrastructural observations are reflected by changes in the immunofluorescence

pattern when anti–type VII antibodies are used for staining of the skin. In normal individuals, type VII collagen epitopes are readily evident in a linear pattern at the dermal–epidermal junction, whereas in generalized HS-RDEB patients the immunostaining is entirely negative. In DDEB, the immunostaining reveals a near-normal pattern and intensity, whereas in M-RDEB, the staining can be attenuated, although clearly positive. These ultrastructural and immunofluorescent findings suggested that type VII collagen was a candidate gene for mutations in the dystrophic forms of EB. This suggestion was subsequently strengthened by demonstration of genetic linkage between the *COL7A1* locus on chromosome 3p21 and both dominantly and recessively inherited forms of DEB.

Subsequent cloning of the human type VII collagen cDNA sequences allowed us to deduce the normal structure of type VII collagen polypeptides. Elucidation of the intron–exon organization of the entire human type VII collagen gene has revealed that the gene is highly complex, consisting of a total of 118 exons. However, *COL7A1* is relatively compact, and the exons are contained within ~32 kb of human genomic DNA. The elucidation of the intron–exon organization of COL7A1 has provided necessary information to undertake mutation analysis of this gene in families with DEB. In fact, specific mutations have now been disclosed in over 300 kindreds with different forms of DEB.

The wealth of information on specific mutations has allowed us to establish genotype–phenotype relationships in different forms of DEB. In normal skin, type VII collagen molecules form antiparallel dimers that associate through their overlapping carboxy-terminal ends (Fig. XX-4). This association is stabilized by interchain disulfide bonds, and such stable type VII collagen dimer molecules laterally aggregate to form anchoring fibrils. Thus, following the synthesis of type VII collagen, several critical steps are required for proper assembly of

anchoring fibrils. Consequently, mutations affecting the synthesis of type VII collagen at the transcriptional or translational level, or those interfering with its supramolecular assembly to anchoring fibrils can result in DEB phenotype (Fig. XX-4).

GENOTYPE/PHENOTYPE CORRELATIONS IN DEB

Severe, Mutilating HS-RDEB: Premature Termination Codon Mutations in COL7A1

In most HS-RDEB patients the consistent genetic lesion is a premature termination codon (PTC) in both alleles of the affected individual. The major effect of a PTC mutation is reduction in mRNA abundance as a result of nonsense-mediated mRNA decay. This phenomenon is coupled to the splicing process itself, since the levels of unspliced, heteronuclear RNA (hnRNA) are equivalent for both the mutant and wild-type alleles, and the decay is evident only upon comparison of processed mRNA. The PTCs result in perturbations in synthesis of type VII collagen mRNAs at the transcriptional level, which are then unable to provide templates for translation of functional polypeptides. Even if the mutant allele containing a PTC is expressed at reduced levels, the translated protein is truncated at its carboxy-terminus and is unable to assemble into anchoring fibrils. These interpretations are consistent with the ultrastructural demonstration of complete absence of the anchoring fibrils in HS-RDEB, and negative immunofluorescence for type VII collagen, thus explaining the extreme fragility of the skin, so characteristic of this phenotype (Fig. XX-4).

Mitis RDEB: Missense and In-Frame Deletion Mutations

In the mitis forms of RDEB, at least one, and in some cases both, alleles encode for a full-

length type VII collagen polypeptide. However, this allele usually contains a missense mutation that can change the conformation of the protein in a manner that the anchoring fibril assembly is perturbed. In some cases, one allele contains a missense mutation or in-frame deletion, whereas the second allele contains a PTC. The net result of the latter combination of mutations is a reduction in mutant RNA from the PTC allele at the transcriptional level, together with a mutation on the second allele, which is transcribed and presumably translated at normal rate, yet is likely to impact on nucleation and assembly of anchoring fibrils (Fig. XX-4). As a result of these more subtle mutations, combined with a PTC on the other allele, mutant full-length type VII collagen molecules may be able to assemble into anchoring fibrils, which are, however, unlikely to be stabilized by disulfide bonding (Fig. XX-4). Thus, these attachment structures, although present, are weakened, resulting in moderately severe fragility of the skin, as observed in M-RDEB.

Dominant Dystrophic EB: Glycine Substitution Mutations in One Allele

In dominantly inherited forms of EB, the recurrent theme of mutations is missense substitutions of glycine residues that occur within the collagenous domain of the type VII collagen molecule, a region characterized by the repeating Gly-X-Y amino acid sequence. These mutated molecules are able to associate with polypeptides synthesized from the normal allele and interfere with their assembly through a mechanism known as dominant-negative interference (Fig. XX-4). The glycine substitutions, therefore, destabilize the collagen triple helix, interfere with the secretion of the molecules, and render them susceptible to intracellular degradation, thus exerting their effect at the posttranslational level. Since type VII collagen is a homotrimer consisting of three identical α1(VII) polypeptides, one out of eight triple-helical molecules

(12.5%) is entirely normal, assuming equal expression of the mutant and wild-type alleles. As a result, some normal type VII collagen homotrimers can be assembled, consistent with ultrastructural demonstration of thin anchoring fibrils, positive immunofluorescence for type VII collagen, and the relatively mild clinical phenotype in DDEB. In addition to the classical forms of DDEB, we have demonstrated glycine substitution mutations in two clinical variants, known as pretibial DEB and the Bart syndrome, proving that these subtypes are allelic to DDEB with mutations in *COL7A1*.

Collectively, the type and combination of mutations are able to predict, in general terms, the clinical severity and natural history of DEB. Since clinical severity represents a continuum in the spectrum of manifestations, the precise nature of the genetic lesions, their positions along the type VII collagen gene, and the dynamic interplay of the two mutant alleles on the individual's genetic background, coupled with environmental influences, will determine the precise phenotype of the patient in DEB.

Revisions in Genetic Counseling in Dystrophic EB

As indicated above, the dystrophic forms of EB can be inherited either in an autosomal dominant or autosomal recessive fashion. The diagnosis of classic HS-RDEB in a patient with severe, mutilating scarring, with clinically unaffected parents, is usually made without difficulty. Similarly, inheritance of a blistering tendency and a relatively mild scarring phenotype, with multiple affected family members in several generations, allows unequivocal diagnosis of dominantly inherited DEB.

The difficulties arise during the diagnosis and ascertainment of the inheritance pattern in patients with a relatively mild phenotype and clinically normal parents. By ultrastructural

analyses, these patients often demonstrate the presence, but a reduced number, of anchoring fibrils, and immunofluorescence shows positive staining for type VII collagen. Consequently, these cases are often diagnosed as dominant DEB, presumed to be caused by a new dominant mutation or reflecting parental germline mosaicism. This diagnosis obviously has implications in terms of genetic counseling of the affected individuals. If their disease is truly a new dominant mutation, the risk of their offspring being affected is one in two (50%). In contrast, in case of a recessively inherited M-RDEB, which is clinically indistinguishable from *de novo* DDEB, the risk of their offspring being affected is low, approximately the same as in the general population, with the exception of consanguineous matings.

Careful determination of the genotype and mutation analysis of several patients with relatively mild disease and ultrastructurally detectable anchoring fibrils with positive immunofluorescence staining for type VII collagen has demonstrated that many of them are compound heterozygotes or have homozygous missense mutations inherited in a recessive manner and therefore the diagnosis is M-RDEB. For example, the first demonstration of type VII collagen mutations in the mitis type of RDEB revealed the presence of a homozygous missense mutation, a methionine-to-lysine substitution (M2798K) at the carboxy-terminal end of the molecule. Similarly, in other cases, a missense mutation in one allele, including a glycine substitution in the collagenous domain, together with a premature termination codon mutation on the other allele, can result in M-RDEB. Finally, our survey of a cohort of over 400 families, in which we have identified distinct COL7A1 mutations, only a limited number of cases appear to be *de novo* dominant mutations at least one of them being derived from the maternal germline.

Based on these considerations, for genetic counseling purposes, it appears appropriate to consider each "new" case as a recessively inherited condition, unless proven to be dominant by

mutation analysis. Reclassification of DEB on the basis of the underlying mutations clearly impacts on the likelihood of the affected individual of having an affected offspring.

APPLICATIONS OF MUTATION ANALYSIS IN PRENATAL DIAGNOSIS OF EB

Precise understanding of the underlying mutations in different forms of DEB has several translational implications in terms of genetic counseling, DNA-based prenatal diagnosis, and gene therapy. Most immediately relevant to the patient care is the development of DNA-based prenatal diagnosis, which can be performed as early as the 10th week gestation through chorionic villus sampling, or through amniocentesis at 15th week on. In the severe dystrophic forms of EB, such analyses can be performed either by direct mutation analyses or by genetic-linkage approaches, recognizing the fact that no evidence for genetic heterogeneity has been disclosed in RDEB. In contrast, in the case of junctional or simplex forms of EB, in which mutations in different genes can result in similar phenotype, prenatal testing has to be based on identification of specific mutations. These approaches have already been applied to DNA-based prenatal diagnosis in over 150 families at risk for recurrence of the severe forms of EB, mostly HS-RDEB and H-JEB. Efforts are currently underway to establish noninvasive prenatal testing by detection of mutations in fetal DNA present in the maternal circulation during the early stages of gestation. The genetic information also provides the basis for development of preimplantation genetic diagnosis through blastomere analysis, a technological advance that would obviate the necessity of termination of the pregnancy in case of an affected fetus, if elected.

GENE THERAPY APPROACHES FOR EB

Precise understanding of the underlying mutations, and subsequent demonstration of the functional consequences of such mutations at the mRNA and protein levels, are obligatory prerequisites for the development of gene therapy approaches for this group of devastating skin diseases. It is conceivable that several forms of EB are realistic targets for genetic therapies of different forms of EB. In fact, progress has been made to transduce keratinocytes cultured from patients with different forms of EB with transgene constructs using either viral vectors or non-viral approaches. However, because of the concern for carcinogenesis due to integration of the viral vectors into the genome, we and others have begun to explore alternative technologies, including direct introduction of genetic material into the skin cells by biolistic particle bombardment ("gene gun"), use of ribozyme-mediated repair of mutant mRNAs by trans-splicing, and application of single-stranded oligonucleotides for targeted gene correction. Thus, establishing the genetic basis of different forms of EB has provided the necessary foundation for development of durable gene therapy approaches to counteract these devastating skin diseases in the future.

Acknowledgments

Carol Kelly assisted in preparation of this manuscript. The original work by the authors was supported by the National Institutes of Health grant P01AR38923 and by The Dystrophic Epidermolysis Bullosa Research Association.

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FIGURE LEGEND

Fig. XX-1. The complexity of the cutaneous basement membrane zone, and classification of epidermolysis bullosa. The figure depicts basal keratinocytes at the lower part of the epidermis, which is separated from the underlying papillary dermis by a dermal-epidermal basement membrane. Ultrastructurally recognizable attachment complexes are indicated on the left, while the specific structural components within each layer are indicated on the right. Also, note the level of tissue separation within different subgroups of epidermolysis bullosa as shown on the right. (Modified from Uitto and Christiano 1992).

Figure XX-2. Phenotypic presentation of selected clinical subtypes of epidermolysis bullosa. (A, B) EB Simplex, Dowling-Meara Type: (A) Herpetiform grouped blisters, erosions and crusts in a generalized distribution; (B) Severe diffuse palmoplantar keratoderma; (C) EB with Muscular Dystrophy: Note blisters, erosions and extensive muscle atrophy; (D) Junctional EB, Herlitz Type: Widespread areas of hemorrhagic blisters and denuded skin; (E) Dystrophic EB, Autosomal Recessive Type: Note coexistence of erosions, ulcerations, scarring and milia formation of the skin leading to mitten deformity of the feet. (F) Dystrophic EB, Autosomal Dominant Type: Note nail dystrophy, scarring and milia formation. Photos (A-C) are courtesy of Drs. Kehua Li (Jefferson Medical College) and Dr. Takashi Hashimoto (Kurume University School of Medicine).

Figure XX-3. Physiology and pathophysiology of keratin intermediate filaments (Top Panel). Synthesis of type I and type II keratin polypeptides and their assembly into keratin intermediate filaments are depicted on the left side of the figure. The keratin mRNAs are translated on the ribosomes of epithelial cells, which synthesize these keratins (I). In the

cytoplasm, a type I and a type II keratin polypeptide align in parallel and register (II) and oligomerize to obligate heterodimers (III). Pairs of heterodimers align in an antiparallel, mostly overlapping fashion to tetramers (IV), which subsequently polymerize to elongated chains packed into keratin intermediate filaments (V). The presence of a mutation in a keratin gene can lead to different pathological processes depicted on the right side of the figure. For example, mutations that introduce a premature termination codon or affect mRNA splicing result in the synthesis of truncated polypeptides. Subsequent nonsense-mediated mRNA decay or enhanced degradation of the truncated polypeptide results in absence of the mutant protein and lack of formation of corresponding heterodimers and KIF, usually associated with severe disease (EBS-DM) (I). Alternatively, the truncated or altered keratin polypeptides can prevent the heterodimer formation and KIF assembly (II). In autosomal dominant keratin disorders, the majority of mutations result in non-conservative amino acid replacements at sites of high sequence conservation (helix boundaries) (III). The mutant keratin polypeptides interfere in a dominant negative manner with head-to-tail interactions and proper alignment of heterodimers (IV) as well as elongation and lateral packing during KIF assembly (V), thus producing a severe phenotype. Mutations with a less severe phenotype reside outside the helix boundaries and may have a more subtle effect on KIF assembly or affect keratin phosphorylation or interaction with other proteins.

Schematic diagram of a keratin protein depicting the structural domains and common mutation sites in EBS (Bottom Panel). The non-helical head domain consists of E1, V1 and H1 (only in type II keratins). The α-helical rod domain is composed of 4 segments, 1A, 1B, 2A and 2B (with stutter-S), which are interrupted by non-helical linker domains L1, L12 and L2. H2 (only in type II keratins), V2 and E2 form the non-helical tail domain. The highly conserved

helix initiation and termination motifs, which are mutational hot spot regions, are striped. Common sites of dominant mutations and their EBS phenotypes are depicted above the diagram, while the location of recessive mutations is shown below. The height of bars reflects the relative frequency of mutations. White symbols: EBS-MP; Dashed symbols: EBS-WC; Black symbols: EBS-DM; Dotted symbols: EBS-K. The nature of mutations is indicated by the symbols: bars represent missense mutations, triangles represent nonsense and frameshift mutations leading to premature termination codon, and stars represent splice site mutations.

Fig. XX-4. The physiology and pathology of type VII collagen. Synthesis of proα1 (VII) collagen polypeptides and their assembly into anchoring fibrils is depicted on the left side of the figure. The mRNA, ~9 kb in size, is translated on the ribosomes of cells, such as basal keratinocytes, synthesizing type VII collagen (I). Within the intracellular space (EC), two of these triple-helical type VII collagen molecules align into an anti-parallel tail-to-tail orientation with overlapping carboxy termini (IV). The molecules are processed by proteolytic removal of the NC-2 domains (•), and the association of the dimer is stabilized by disulfide bonding (V). Subsequently, a large number of the dimer molecules laterally assemble into anchoring fibrils which contain at both ends intact amino-terminal NC-1 domains with adhesive properties (VI).

In the presence of genetic lesions in COL7A1, type VII collagen pathology can manifest as different variants of EB. For example, premature termination codon mutations (PTC) result in synthesis of truncated polypeptides unable to form anchoring fibrils, causing severe Hallopeau-Siemens type of recessive dystrophic EB (HS-RDEB). In a milder autosomal recessive form, known as the mitis variant (M-RDEB), missense mutations either homozygous or compound heterozygous state, or in combination with a PTC *in trans* prevent the assembly of type VII collagen dimers. In case of dominant dystrophic EB (DDEB), glycine substitution mutations

affecting the collagenous domain of type VII collagen interfere with the packing of the anchoring fibrils (Modified from Uitto and Pulkkinen 2000).